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Peptide Prodrugs: Using Host Defence Peptides as Therapeutic Anticancer Agents

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Peptide Prodrugs: Using Host Defence Peptides as Therapeutic Anticancer Agents

RCSI

This thesis is submitted to the National University of Ireland for the degree of Doctor of Philosophy

By

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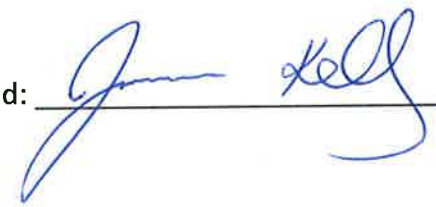
Under the supervision of Dr Marc Devocelle

Dedication

To my Parents and Brother

Declaration

I declare that this thesis, which I submit to Royal College of Surgeons for examination in consideration of the award of a higher degree Doctor of Philosophy, is my own personal effort. Where any of the content presented is the result of input of data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

Signed: 

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Publications and Presentations:

Journal/Poster/Oral Presentations:

Graeme Kelly, Marc Devocelle, Host Defence Peptides and their Therapeutic Applications; Oral Presentation, 2nd Irish Peptide workshop, University of Ulster Coleraine.

Graeme Kelly, Marc Devocelle, Using Host Defence Peptides as Therapeutic Anticancer Agents; Oral Presentation, RCSI Research Day 2012, Royal College of Surgeons.

Graeme Kelly, Marc Devocelle, Using Host Defence Peptides as Therapeutic Anticancer Agents; Oral Presentation, Irish Chemistry Colloquium 2011, University College Dublin.

Graeme Kelly, Agnieska Folytyn-Arfa Kiab, Siobhan McClean, Marc Devocelle. Polymeric Drug Combination Therapy Based on Host Defence Peptides Targeting Cancer Cells; Poster Presentation, Irish Chemistry Colloquium 2011, University College Dublin

Graeme Kelly, Tanya Smith, Marcus Sims, Marc Devocelle. A Dual-Acting Heterodimeric Peptide; Abstract published in Journal of Biopolymers Peptide Science, (2011) 96; 4, Page 477

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Graeme Kelly, Marcus Sims, Marc Devocelle. The use of a heterodimeric peptide as an anticancer agent; Poster Presentation, Centre of Synthesis and Chemical Biology, Trinity College Dublin 2010.

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Summary

According to the World Health Organization (WHO), Cancer is the leading cause of death worldwide, accounting for around 13% of all deaths in the world annually.

The design of cancer chemotherapy has become increasingly sophisticated and the treatment of cancer has become increasingly specific with concentration focused on molecular targets. At the moment, there is still no cancer treatment that is 100% efficient against wide spread cancer so there is still a growing demand for the investigation into developing novel anticancer agents.

This project is aimed at developing prodrugs of Host defence peptides (HDPs). HDPs are multifunctional molecular effectors of innate immunity, the first line of defence against infection in multicellular organisms. Their synthetic derivatives also have exciting potential as anti cancer agents.

In the development of the project, three different approaches were taken to explore some of the different targeting mechanisms of drug delivery;

- The first approach involves a dual release prodrug which contains a PEGylated HDP joined by a degradable enzyme peptide linker. On the other side of the PEG is a classical anticancer agent attached by an acid labile functional. These linkers are key to allow the release of the peptide and anticancer agent upon degradation or change in pH conditions, and are suitable for lysosomotropic delivery.

- The second approach is a peptide prodrug which exploits enzyme degradable linkers and allows for targeted delivery against various types of cancer cells.

- The third approach is a delivery technique using a HDP as a vector to deliver a pro apoptotic peptide sequence into the cancer cell, as well as an active anticancer agent, contributing to the overall activity of the dimer.

Glossary

Table of amino acids and Protection groups

Name	3 letter code + protection	1 letter code 'L' Configuration	1 letter code 'D' Configuration
Histidine	Fmoc-His(trt)-OH	H	h
Arginine	Fmoc-Arg(Pbf)-OH	R	r
Lysine	Fmoc-Lys(Boc)-OH	K	k
Alanine	Fmoc Ala-OH	A	a
Valine	Fmoc-Val-OH	V	v
Phenylalanine	Fmoc-Phe-OH	F	f
Leucine	Fmoc-Leu-OH	L	l
Isoleucine	Fmoc-Ile-OH	I	i
Methionine	Fmoc-Met-OH	M	m
Tryptophan	Fmoc-Trp(Boc)-OH	W	w
Proline	Fmoc-Pro-OH	P	p
Glycine	Fmoc-Gly-OH	G	g
Serine	Fmoc-Ser(tBu)-OH	S	s
Threonine	Fmoc-Thr(tBu)-OH	T	t
Cysteine	Fmoc-Cys(Trt)-OH	C	c
Asparagine	Fmoc-Asn(Trt)-OH	N	n
Glutamine	Fmoc-Gln(Trt)-OH	Q	q
Aspartic acid	Fmoc-Asp(OtBu)-OH	D	d
Glutamic Acid	Fmoc-Glu(OtBu)-OH	E	e
Tyrosine	Fmoc-Tyr(tBu)-OH	Y	y

List of Abbreviations

4t1.2 Luc	Breast cancer cell line
A2780P	Ovarian Cancer cell Line
ABC	ATP Binding Cassettes
Ac	Acetyl
Ahx	Hexanoic acid
AMPs	Antimicrobial peptides
APAF1	Apoptotic peptidase activating factor 1
ATP	Adenosine triphosphate
B16-BL6	Mouse melanoma
BAD	BH3 only protein
BAX	BH3 only protein
Bcl2	B-cell lymphoma 2
Benzyl PEG	BZ-PEG-OH
BH Domain	Bcl-2 homology domain
BH1	Bcl-2 homology domain-1
BH2	Bcl-2 homology domain-2
BH3	Bcl-2 homology domain-3
BH4	Bcl-2 homology domain-4
BID	BH3 interacting-domain
BIM	BH3 only protein
BMAP-28	Bovine myeloid antimicrobial peptide
BOC	*tert*-Butyloxycarbonyl

BOP	Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate
BTC PEG	PEG Benzotriazole
CAPs	Cationic Antimicrobial Peptides
CCRF-SB	Acute Lymphoblastic Leukemia cell line
CD	Circular Dichroism
$CDCl_3$	Deuterated Chloroform
CEM-VLB	Human Lymphoblastic Leukemic cells
CM-PEG	Carboxymethyl-PEG
D_2O	Deuterated Water
DCC	*N,N'*-Dicyclohexylcarbodiimide
DCM	Dichloromethane
DIEA/DIPEA	*N,N*-Diisopropylethylamine
DMAP	4-Dimethylaminopyridine
DMEM	Dulbecco's Modified Eagle Medium
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
DTNP	2,2'-Dithiobis(5-nitropyridine)
ECM	Extra Cellular Matrix
EDT	1,2-Ethanedithiol
EDTA	Ethylenediaminetetraacetic acid

EPR	Enhanced permeability and retention
ESI	Electro-Spray ionization
Fas	Tumour necrosis factor receptor superfamily member 6
FBS	Fetal Bovine Serum
FIC	Fractional Inhibitory
FMOC	9-Fluorenylmethyloxycarbonyl chloride
H2052	Small lung cancer cell line
HATU	1-[Bis(dimethylamino)methylene] 1H-1,2,3-triazolo [4,5-b]pyridinium 3-oxid hexafluorophosphate
HBTU	*N,N,N',N'*-Tetramethyl-O-(1H-benzotriazol-1-yl) uronium hexafluorophosphate
HBV	Hepatitis B virus
hCAP-18	Human cathelicidin antimicrobial protein
HDPs	Host defence peptides
HeLa	Cervical cancer cells
HIV	Human immunodeficiency virus
HMPA	Hexamethylphosphoramide
HOAt	1-Hydroxy-7-azabenzotriazole
HOBt	Hydroxybenzotriazole
HPV	Human papillomavirus
IAP	Inhibitors of apoptosis
Imd	Immune deficiency
IR	Infra Red

LFCIN-B	Lactoferricin B
LL-37	Antimicrobial peptide
mAbs	Monoclonal antibodies
MBHA	(4-methyl)benzhydrylamine-resin
Mdm2	Mouse double minute 2 homolog
MDR	Multidrug resistance
MDR1	Multidrug resistance-1
MeOD	Deuterated Methanol
MESO	Small lung cancer cell line
MMP	Matrixmetalloproteinase
MMP-2	Matrixmetalloproteinase-2
MMP-9	Matrixmetalloproteinase-9
mPEG	methoxypoly(ethylene glycol)
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl) -2-(4-sulfophenyl)-2H-tetrazolium)
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide
NHS	*N*-Hydroxysuccinimide
NMP	*N*-Methyl-2-pyrrolidone
NMR	Nuclear magnetic resonance
O*t*Bu	*t*-butyl ester
P13k	Phosphatidylinositol 3-kinase
$P13k_p85_\alpha$	Phosphatidylinositol 3-kinase subunit
PAP	Papanicolaou test

PC	Phosphatidylcholine
PDI	Polydispersity Index
PEG	Poly(Ethylene Glycol)
PEG-IA	PEG iodoacetate
PEG-MAL	PEG Maleimide
PEG-OPSS	PEG Orthopyridyl Disulfide
PEG-VS	PEG-Vinylsulfone
PEP-1	Cell-Penetrating Peptide
PGA	Poly Glutamic Acid
P-gp	P-glycoprotein
PLGa	Poly(Lactic-co-Glycolic Acid)
pNpys	5-Nitro-2-pyridinesulfenyl
PR-39	Porcine Host Defence Peptide
PS	Phosphatidylserine
PTD	Protein Transduction Domain
PUMA	BH-3 only protein
PyBOP	Benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate
Raji	Human hematopoietic cell line
REN	Small lung cancer cell line
RGD	Arg-Gly-Asp
RPMI	Roswell Park Memorial Institute medium
SB-37	Antimicrobial peptide

SC PEG	PEG-succinimidyl carbonate
SCM	succinimidylester of CM-PEG
SEC	Size exclusion chromatography
siRNA	Small interfering Ribonucleic acid
SKVO3	Ovarian cancer cell line
SMANCS	Styrene maleic anhydride neocarzinostatin co-polymer
SPPS	Solid Phase Peptide synthesis
SS-PEG	PEG-succinimidyl succinate
TA	Thioanisole
TAT	Trans-Activator of Transcription
*t*BU	*tert*-Butyl
TCP-PEG	Trichlorophenol carbonate
TFA	Trifluroacetic acid
TIPS	Triisopropylsilane
Tm	Transmembrane domain
TNF	Tumour necrosis factor
TRAIL	TNF-related apoptosis ligand
TRL	Toll-like Receptor
TSU	Bladder carcinoma cells
U937	Human histiocytic lymphoma
UV	Ultra Violet
VIA	Visual inspection with acetic acid
WHO	World Health Organisation

βAla	Beta-Alanine

Chapter 1

General Introduction

1 Introduction

1.1 Cancer

1.1.1 What is Cancer?

Cancer is the generic term used to define a group of diseases that can affect any part of the body. The word cancer is derived from the Latin word for "crab" which describes how the blood vessels of a tumor look[1]. Cancer is the result of uncontrolled cell growth[2]. Our bodies are made up of trillions of cells all working together. For cancer to develop, one of those cells has to stop regulating properly and ignore the normal process of cell death[1]. Cancer cells still share a large number of properties and identical needs as normal cells but they come independent of the controls that make our bodies run smoothly. Every case of cancer is unique, with its own set of genetic changes and growth properties. Some cancers can grow quickly while others can take years to become clinically significant. Different cases of cancer can even apply to the same organ which makes the problem of targeting and treating cancer so difficult. One defining feature of cancer is the rapid creation of abnormal cells that grow beyond their usual boundaries and which can then invade adjoining parts of the body and spread to other organs. This process is referred to as metastasis. Metastases are the major cause of death from cancer[3]. Despite the different types and different cases of cancer, all cancers do however contain common similarities. It is within the similarities of cancer cells that we have the best chance of finding an understanding, diagnosis and treatment of cancer[4].

1.2 Cancer in society

1.2.1 Global Cancer Statistics

The total amount of cancer related deaths is collected and published each year by the World Health Organisation (WHO)[3]. Cancer is now the leading cause of death worldwide, accounting for 7.6 million deaths in 2008. This accounts to around 13% of all deaths on the planet in that year, Table 1.1.

Table 1.1: Cancer types and deaths worldwide

Cancer Type	Deaths Worldwide
Lung	1.4 Million
Stomach	0.74 Million
Liver	0.7 Million
Colorectal	0.61 Million
Breast Cancer	0.43 Million

With 12.7 million new cases of cancer each year and 7.6 million of cancer related deaths reported in 2008 worldwide, 56% of these cases have occurred in the economically developing world accounting for 64% of the overall total cancer mortality[5], Figure 1.1. About 30% of cancer deaths are due to five leading behavioural and dietary risks: high body mass index, low fruit and vegetable intake, lack of physical activity, tobacco use and alcohol use. Ageing is another fundamental factor for the development of cancer. Cancer incidence rises dramatically with age, most likely due to a build-up of risks for specific cancers that increase with age[5]. The overall risk accumulation is combined with the tendency for cellular repair mechanisms to be less effective as a person grows older. Knowledge about the causes of cancer, and interventions to prevent and manage the disease is now extensive. The incidence of cancer can be reduced by implementing evidence-based strategies for cancer prevention, early detection of cancer and management of patients with cancer. There are many prevention strategies such as increased

avoidance of behavioural and dietary risks, vaccination against human papilloma virus (HPV) and hepatitis B virus (HBV), control of occupational hazards and reduced exposure to sunlight[6].

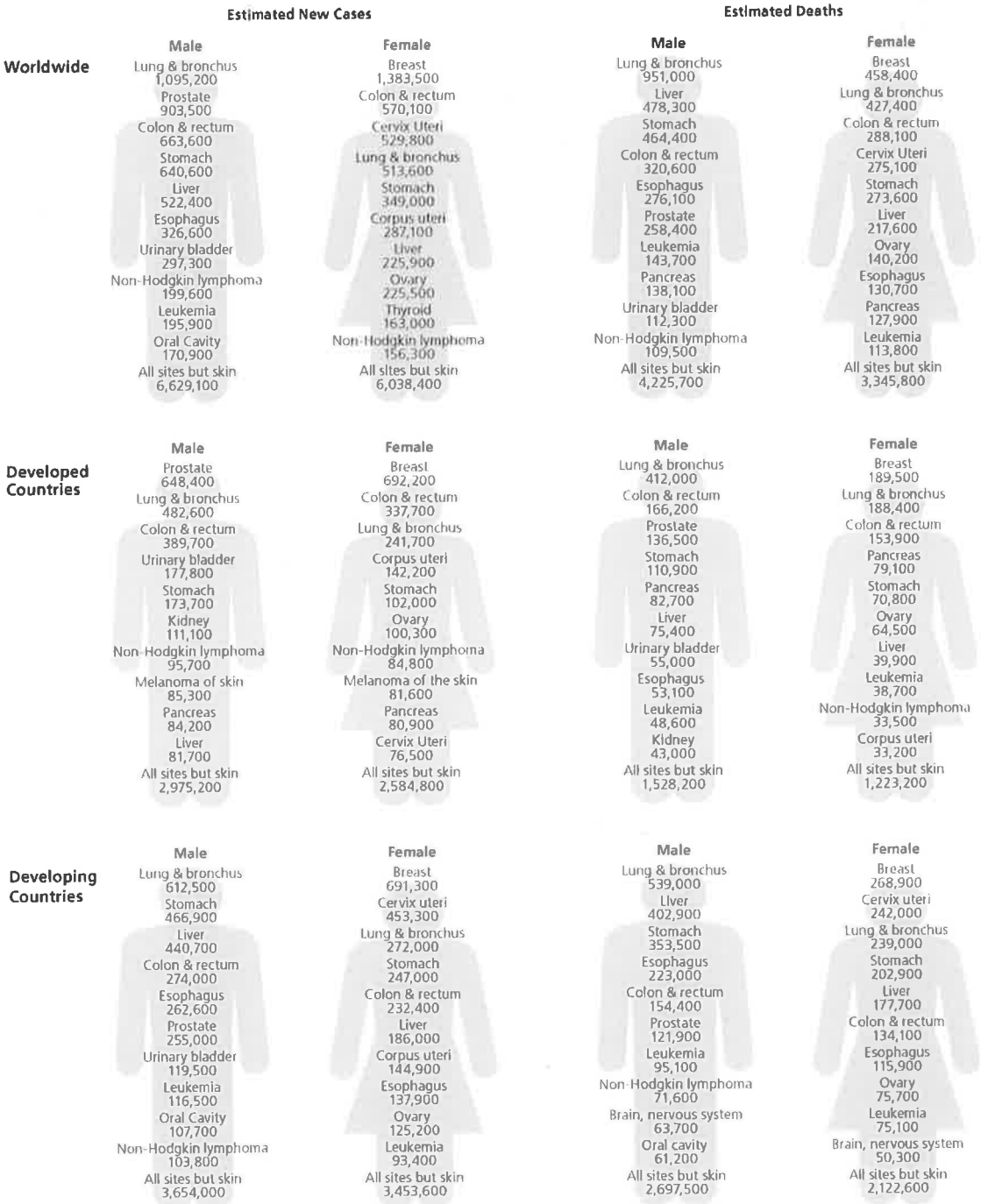


Figure 1.1: Different cancers and trends between worldwide, developing and developed countries; estimate of worldwide burden of cancer in 2008: Fom Globocan 2008.

1.2.2 Early detection and screening of cancer

Cancer mortality can be reduced if cases are detected and treated early. There are two components of early detection efforts. The awareness of early signs and symptoms of cancer is important. This is especially the case in cancers such as cervical, breast and oral cancers. Early detection can help to facilitate diagnosis and treatment before the disease becomes advanced. Early diagnosis programmes are particularly relevant in low-resource settings where the majority of patients are diagnosed in very late stages. The systematic application of a screening test in an asymptomatic population is very important to try and detect cancers at their earliest stages. It aims to identify individuals with abnormalities suggestive of a specific cancer or pre-cancer and refer them promptly for diagnosis and treatment. Screening programmes are especially effective for frequent cancer types that have a screening test that is cost-effective, acceptable and accessible to the majority of the population at risk. Examples of screening methods are; visual inspection with acetic acid (VIA) for cervical cancer in low-resource settings, Papanicolaou (Pap) test for cervical cancer and mammography screening for breast cancer[7,8].

1.2.3 Treatments

Treatment is the series of interventions, including psychosocial support, surgery, radiotherapy and chemotherapy that is aimed to cure the disease or considerably prolong life while improving the patient's quality of life. Some of the most common cancer types, such as breast, cervical, oral and colorectal have higher cure rates when detected early and treated according to best practices. Some cancer types, even when disseminated, such as leukaemias and lymphomas in children, and testicular seminoma, have high cure rates if appropriate treatment is provided[9].

1.2.4 Palliative care

Palliative care is treatment to relieve, rather than cure, symptoms caused by cancer. Palliative care can help cancer patients live more comfortably. It is an urgent humanitarian need for people worldwide with cancer and other chronic fatal diseases. It is particularly needed in places with a high proportion of patients in advanced stages where there is little chance of cure. Relief from physical, psychosocial and spiritual problems can be achieved in over 90% of advanced cancer patients through palliative care. Effective public health strategies, comprising of community and home based care are essential to provide pain relief and palliative care for patients and their families in low-resource settings. Improved access to oral morphine is desirable for the treatment of moderate to severe cancer pain, suffered by over 80% of cancer patients in terminal phase[10,9].

1.3 Current approaches to cancer therapeutics

The hallmarks of cancer are the biological processes that characterize all tumors. These form the foundation of modern therapeutic approaches. In the past decade there have been 55 therapeutics approved with over half of them targeting specific proteins. Nearly three quarters of the 55 are based on functional antagonism of cancer cell survival and growth mechanisms[11].

1.3.1 Dysregulated proliferation

Dysregulated proliferation is the signal feature of cancer and oncology drug discovery has focused intently on the different phases of growth control. Converting knowledge of basic biology of cell division, facilitated greatly by evolutionary conservation of many fundamental aspects of the cell cycle, has brought about the development of a large number of anti-proliferative therapeutics. There are a range of drugs to combat cancer in this method from traditional cytotoxics to inhibitors of specific proteins during different stages of mitosis (neocytotoxics). Many of the genes that encode these antiproliferative

targets are highlighted by somatic mutations that provide direct evidence for their role in the genesis of cancer[11].

1.3.2 Invasion and migration

Invasion and migration are two distinct characteristics of tumor cells that are closely related and are responsible for metastasis and tumor spread. There are a few anti metastatic targets including metalloproteases and signal-transduction inhibitors[12]. However, to date none have been approved for drug administration. Antimetastatic agents are considered to be most effective during the early onset of cancer, a setting in which new cancer therapeutics are rarely tested, so this may be a barrier to their successful development[11].

1.3.3 Apoptosis

Apoptosis is one of the key mechanism that ensure cells are grown in their native surroundings. Normal cells that grow in an abnormal environment can undergo a process known as 'suicide' or programmed cell death[13]. This process guarantees proper cell development and protects against cancer and autoimmunity. However, tumor cells have the ability to ignore the stimuli coming from the apoptotic pathway and ultimately become resistant to a broad set of stressors, including drug therapy. There are several experimental therapeutics to target these apoptosis pathways such as; TRAIL and inhibitors of bcl2, mdm2 and IAP. The aim of targeting these pathways is to selectively activate cell death in tumor cells[2].

1.3.4 Immortality

Immortality is a universal feature of malignant cells and only stem cells possess this same trait. All other normal dividing cells divide a finite number of times, differentiate or senesce and die. Other than telomerase, the enzyme that lengthens telomeres, few targets have been uncovered with specific roles in cellular lifespan and so far no drugs have targeted this mechanism[14].

1.3.5 Angiogenesis

Angiogenesis has been the cornerstone of development in cancer drugs and it has been highly investigated and exploited for clinical benefit in the past decade. Tumors need a certain amount of nutrients to survive which are carried in the blood. Predicted broad activity and potential add-on to other drugs recommend this approach for medical and commercial reasons[15].

1.4 What are the limitations in cancer therapies?

Oncology has one of the most poorest records of investigational drugs in development with success rates that are up to three times lower than other diseases, for example cardiovascular diseases. The lethal nature of cancer persists and even with billions of dollars of public and private investments there seems to be only slight increments in overall treatment outcomes.[16] Chemotherapeutic drugs play a pivotal role in the treatment of many different types of cancers. They aren't without drawbacks as chemotherapy agents generally attack rapidly dividing cancer cells, both neoplastic and healthy proliferating cells, which result in side-effects of chemotherapy.[17] Chemotherapy can have a lot of undesirable side-effects ranging from nausea and vomiting to myelosuppression and thrombocytopenia.[18] Cancer cells have also developed a resistance to many of the anticancer drugs which in turn greatly reduces their therapeutic benefits.[19]

1.4.1 Cancer Drug Resistance

The design of cancer chemotherapy has become increasingly sophisticated and the treatment of cancer has become increasingly specific with programmes aimed at molecular targets derived from studies of the oncogenes and tumor suppressors known to be involved in the development of human cancers. There is still no cancer treatment that is 100% efficient against widespread cancer.[19] This increased

specificity of cancer treatment, from the use of general cytotoxic agents such as nitrogen mustard used in the 1940s, to the development of natural-product anticancer drugs in the 1960s such as *Vinca* alkaloids and anthracyclines, which are more cytotoxic to cancer cells than normal cells, to the use of specific monoclonal antibodies and immunotoxins targeted to cell surface receptors and specific agents that inactivate kinases in growth-promoting pathways, has improved the response rate in cancer and reduced side-effects of anticancer treatment but has not yet resulted in cure of the majority of patients with metastatic diseases[20,21]. A study of the mechanisms by which cancers can elude treatment has yielded a wealth of information about why these therapies fail and is beginning to yield valuable information about how to circumvent drug resistance in cancer cells and/or design agents that are not subject to the usual means of resistance.

1.4.2 How Cancer Cells Elude Chemotherapy

Cancer cells can escape the effects of chemotherapy for two general reasons. The first are host factors and the specific genetics of cancer cells while the second are epigenetic alterations in cancer cells[19]. Host factors include: the poor absorption, rapid metabolic degradation and excretion of the drug, resulting in low serum levels; the poor tolerance to the effects of the drug, resulting in reducing the optimum levels of the drug; the inability to deliver drugs to solid bulky tumours or biological agents with high molecular weight; the limited ability of monoclonal antibodies and immunotoxins to penetrate cell membranes[22]; alterations in the host-tumour environment that can affect response of the tumour, including local metabolism of the drug by non-tumour cells. Also present are unusual features of the tumour blood supply that may affect transit time of drugs within tumours and the way in which the cancer cells interact with each other and the interstitial cells from the host[23]. There are many different mechanisms by which cancer cells, grown in tissue culture, have become resistant to anticancer drugs. Some of these mechanisms include; a loss of a cell surface receptor or a transporter for a drug; a specific metabolism of a drug; or alteration by mutation of the specific target of a drug. All of these mechanisms occur for antifolates such as methotrexate, resulting

in resistance to only a small number of related drugs[24]. In these cases drug combinations are used which use different mechanisms of entry into cells and modulate different targets, allowing for effective chemotherapy and higher cure rates in patients. However, some cancer cells become resistant to many types of drugs simultaneously; this phenomenon is known as multidrug resistance (MDR). Multidrug resistance results from changes that limit accumulation of drugs by eliminating uptake, enhancing efflux or affecting membrane lipids. All of the above changes can affect the process of apoptosis activated by anticancer drugs. The activation of general response mechanisms to detoxify and repair cell damage, as well as changes to cell cycles and checkpoints that render cells relatively resistant to the cytotoxic effects of drugs on cancer cells can also occur[19].

1.4.3 P-glycoprotein and its influence on multidrug resistance against anticancer drugs

The major mechanism of multidrug resistance comes from the energy-dependent drug efflux pump known as P-glycoprotein (P-gp). It is a product of the *MDR*1 gene in humans and it is found amongst a large family of ATP-dependent transporters known as ATP-binding cassettes (ABC) family[25]. They are not only involved in the efflux of drugs but also in the movement of nutrients in and out of the cell. However P-gp is over-expressed in many types of human cancers[26]. It can detect and bind a large variety of hydrophobic natural products as they enter the plasma membrane. These drugs include many of the commonly used natural product anticancer agents such as doxorubicin, daunorubicin, vinblastine, vincristine, and taxol, as well as many commonly used pharmaceuticals ranging from antiarrhythmics and antihistamines, to cholesterol lowering statins and HIV inhibitors. When these drugs bind to P-gp, this results in the activation of one of two ATP binding domains and the hydrolysis of ATP, causing a change in the shape of P-gp which results in the release of the drug into the extracellular space and out of the cell[27].

1.4.4 Peptides as anticancer agents

Most of the anticancer agents currently in use are based on the alkylating agents, anti-metabolites and natural products, with DNA being the main target for anticancer drug development[28]. This has resulted in the production of many successful chemotherapeutic agents. However there have been many problems associated with these chemotherapeutics which result in undesirable side-effects. Identification of targets such as cancer specific proteins on the cell membrane would allow for a more targeted approach to chemotherapy. This has been tested to some degree with monoclonal antibodies (mAbs). However, their low tissue penetration and poor cellular uptake *in vivo* is a major drawback to their clinical application [29]. In contrast to some mAbs, peptides are nearly invisible to the immune system and are expected to cause minimal or no side-effects[29]. Although therapeutic peptides entered the market twenty years ago, they witnessed a greater focus of research during the past decade. The growth analysis firm Frost and Sullivan described in 2011 that there are roughly 40 peptide drugs on the market today, *e.g.* oxytocin, cyclosporin enfuvirtide and goserlin with around 270 in clinical trials and over 400 in advanced preclinical trials. The number of peptide-based therapies is therefore predicted to grow exponentially over the coming years. Although the cost of peptide therapeutics is still a major drawback for large scale industrial production, the cost reduction in the materials for peptide synthesis and the improvements in manufacturing processes and delivery systems triggered a revival of interest. Therapeutic peptides are thought to be one of the most attractive options in medicine at the moment. This is due to their low toxicity, high specificity and much lower molecular weight than antibodies and proteins in general. They can also overcome the major problem of multi-drug resistance met by current chemotherapies[17].

1.5 Peptides of the innate immune system

1.5.1 Introduction

Antimicrobial peptides (AMPs) are evolutionarily ancient weapons. Their widespread distribution throughout the animal and plant kingdoms suggests that antimicrobial peptides have served a fundamental role in the successful evolution of complex multicellular organisms.[30] They were originally discovered around 30 years ago in plants, insects and amphibians[31]. Within these peptide sequences they contain a wide defence mechanism that is non-specific and has a wide spectrum of activity against many different types of cancer, bacteria and viruses. They are the first line of defence against infection for many species in nature. There are two main groups of AMPs and they can be classified by how they are expressed, ribosomal and non-ribosomal synthesised peptides. The non-ribosomal assembled sequences are only found to be produced by fungi and bacteria[32,33]. The ribosomal assembled sequences are specific to their hosts and are the main molecular mediators of innate immunity. Despite their ancient lineage, they have remained effective defensive weapons, confounding the general belief that bacteria, fungi and viruses can and will develop resistance to any conceivable substance. AMPs target a previously under-appreciated "microbial Achilles heel", a design feature of the microbial cellular membrane which distinguishes broad species of microbes from multicellular plants and animals. The insights provided by a large body of research have spawned considerable commercial effort to create new classes of anti-infective therapeutics.

It has been recently suggested by Hancock *et al.* that the term 'antimicrobial peptide' should be used when direct antimicrobial activity is being examined, and the term 'host defence peptide' (HPD) should be used when referring to anti-infective activity that enhances or modulates the host immune response to infectious agents[34].

1.5.2 Non-Ribosomal Peptides

1.5.2.1 Antimicrobial peptides

Non-ribosomal peptides are a class of non-cationic AMPs produced by bacteria, fungi and streptomycetes[35,36]. They are illustrated by penicillin and cephalosporins[32]. They are produced by large multi-enzyme systems, known as thiotemplates, the non-ribosomal peptide synthetases, which contain several modules, with each module consisting of at least three domains involved in selection, activation and sometimes some modification[37]. They work primarily by either post-translational modification outside the cell giving rise to the active form of the peptide or excretion of an inactive pro-peptide which is proteolytically activated outside the cell to release the activte peptide. An example of this can be seen in the biosynthesis of gramicidin S[37].

They differ from host defence peptides as they don't have a specific make-up of amino acids and contain a primary structure that is not constant. They include the acidic lipopeptides, glycopeptides, peptides with miscellaneous amino acid content and peptides that are constrained by several loops formed by heterocyclization, cross-linkages and macrocyclization[37].

The non-ribosomal amino acids contain more than 100 different types of building blocks; this is in contrast to ribosomal building blocks which only contain 20 amino acids. They also consist of D-amino acids, *N*-terminal fatty acids, *N*- and *C*-termini methylated residues, *N*-formylated residues, heterocyclic elements, glycosylated amino acids and phosphorylated residues[37]. A number of these peptides, for example daptomycin and vancomycin, have been clinically approved as antibiotics[35].

1.5.3 Ribosomal Peptides

1.5.3.1 Host defence peptides

To date, there have been more than 1,000 HDPs characterised or predicted from different sources, including natural, synthetic and also hybrid modified sequences[38]. They can be found in virtually every living species from unicellular to multicellular organisms, including bacteria, fungi, plants, crustaceans, tunicates, insects, fishes, amphibians, birds and mammals[38]. Invertebrates are a class of animals that don't contain a backbone. Insects make up the majority of the invertebrate class, which are the most expansive class of all living species. There are approximately 800,000 different species, this in turn represents about 80 % of all the animal species on Earth[39]. For all invertebrates, the innate immunity mechanisms are their only defence against infection. Insects for example can produce up to of 10 to 15 different peptides as part of their innate immunity. These peptides form different types of secondary structures, *i.e.* linear and alpha-helical, disulfide bridged, linear non-alpha-helical and rich in one amino acid and finally β-sheet containing peptides, with each of them displaying different activities against many invading pathogens[39]. AMPs/HDPs from invertebrates have amongst the highest antimicrobial activity and anticancer activity, respectively. Although they are produced constantly and are active at sites exposed to bacteria, they are also induced upon infection. Their production is triggered by toll like receptors (TLRs) or the immune-deficiency (Imd) pathway of phagocytosis in response to infection. They are produced by hemocytes and in fat tissue of invertebrates which would be very similar to the liver in vertebrate species[40]. They are released systemically in high concentrations nearly 130 times faster than antibodies in vertebrates. This in turn allows them to compete efficiently with the multiplication rate of bacteria[39].

Each group of antimicrobial peptides can be easy identified by their secondary structure or by the presence of specific amino acid sequences. HDPs consist of short polycationic sequences (12 to 50 amino acid residues) with a high content of hydrophobic residues. Not all peptides are polycationic, some anionic peptides are also known to exist[41]. Apart from these common features, they have very limited

sequence homology and are divided into several groups according to their secondary and tertiary structures and also to the presence or absence of specific residues, such as cysteine, tryptophan, proline and histidine[41c].

1.5.3.1.1 Linear α-helical peptides

Linear α-helical peptides (Figure 1.2 (E)) are a group of peptides which display cationic amphipathic helical structures and possess inhibitory activities generally against bacteria. Many peptides of this group have been classified as "pore forming" such as alamethicin, cecropin, PLGa, magainin, melittin and mastoparan. They are short (less than 40 amino acids in length), rich in hydrophobic and basic amino acids and have no disulfide bonds. They can adopt an α-helical amphipathic secondary structure under appropriate conditions, which may be essential for their biological properties. These peptides are widely distributed in nature and exhibit broad spectrum antimicrobial, antiprotozoal and hemolytic activities.[42] They have been more recently used as therapeutic anticancer candidates.

1.5.3.1.2 Disulfide bridged defensins

Disulfide bridged defensins (Figure 1.2 (A+D)), are cyclic peptides that predominantly form β-sheets or β-stands due to the presence of two or more disulfide bridges. They contain more residues than most linear peptides, with 33-46 amino acids in their sequences. The latter are similar within an order, but quite unrelated between different ones and exceptions to the common structure, such as peptide chain extension at either the C- or N-terminus, have been identified. Their secondary structure is stabilized by three to four disulfide bridges internally within the peptide sequence. The position of six cysteines and their bridging pattern are conserved among insect defensins, but differ from that of mammalian defensins[39]. Unlike cecropins, they are usually not amidated at their C-termini. Although showing antifungal activity, they are characterized as antibacterial peptides. The β-sheets are stably assembled by either disulfide bonds as in the case of tachyplesins,

defensins, protegrins and gallerimycin or cyclic peptide backbone, as in the case of polymyxin B, trycodines and arenicins.

1.5.3.1.3 Linear peptides rich in one amino acid (Pro, Trp, Arg, His, Gly)

Some linear peptides contain large amounts of one particular amino acid. The majority of them are rich in proline but other peptides with a high percentage of a particular amino acid have also been discovered. Linear proline-rich peptides contain at least 25 % of proline residues, usually found in conserved segments such as Pro-Arg-Pro and Pro-His-Pro[43]. They can be organised in two groups according to their length, 20 residues being the cut-off point for their classification[39]. Drosocin, methnikowin, apidaecin, abaecin, formaecin, lebocin, pyrrhocoricin and metalnikowin are examples of these proline-rich peptides. Indolicidin is found to be highly rich in tryptophan whereas histatin is found to be rich in histidine (Figure 1.2 (F)). Others examples include tritrpticin which is rich in arginine or tryptophan and diptericins and attacins which are highly rich in glycine residues. These peptides are generally found in insects and have been shown to be active against bacteria and fungi. It is for some of these peptides that the distinctive pos-translational modification of serine or threonine residues by *O*-glycosylation can be found. The physiological relevance of this modification remains contradictory, as either an increase or decrease of the MIC has been evidenced *in* vitro for their non-glycosylated counterparts[39]. Their cytotoxic activity occurs at lower rates than for defensins. Short peptide chains are usually exclusively active against Gram-negative bacteria and their uptake can be energy driven[43].

1.5.3.1.4 Beta-sheet folded peptides

The beta-sheet folded peptides (Figure 1.2 (B)) have a β-hairpin or looped configuration due to the presence of a single disulfide bond and/or a cyclization of the peptide chain. Their sequences are short, containing around 20 amino acid residues and they are among the most potent HDPs. They are found to be active against both types of bacteria. They are stored in the granules of hemocytes and in the hemolymph[44]. An example of beta-sheet folded peptide produced by invertebrates is thanatin, a 21-mer sequence containing a single disulfide bond[45], which possesses 40 % sequence similarity with the brevinin family peptide from the frog *Rana brevipoda*. Thanatin possesses the largest spectrum of activity among arthropod peptides. The peptide is active at a maximum concentration of 10 μM and has haemolytic activity[40].

Despite the great diversity of amino acid residues making up the primary structures of HDPs, a few conserved characteristics can be found in these peptides. They possess a length comprised of between 10 to 50 residues, a net positive charge from +2 to +9 and a content of hydrophobic residues higher than 30%[46]. These features allow cationic antimicrobial peptides to adopt general amphipathic secondary structures[46]. These secondary structures can be studied with tools such as NMR which can be exploited to determine the conformation of the peptide in different solvents (generally different proportions of water and trifluoroethanol) or their interaction with structures such as micelles or lipidic membrane mimetics, by solid state NMR. Other methods which has been implemented include circular dichroism (CD) to probe the propensity of the sequence to form secondary structures[41c, 47].

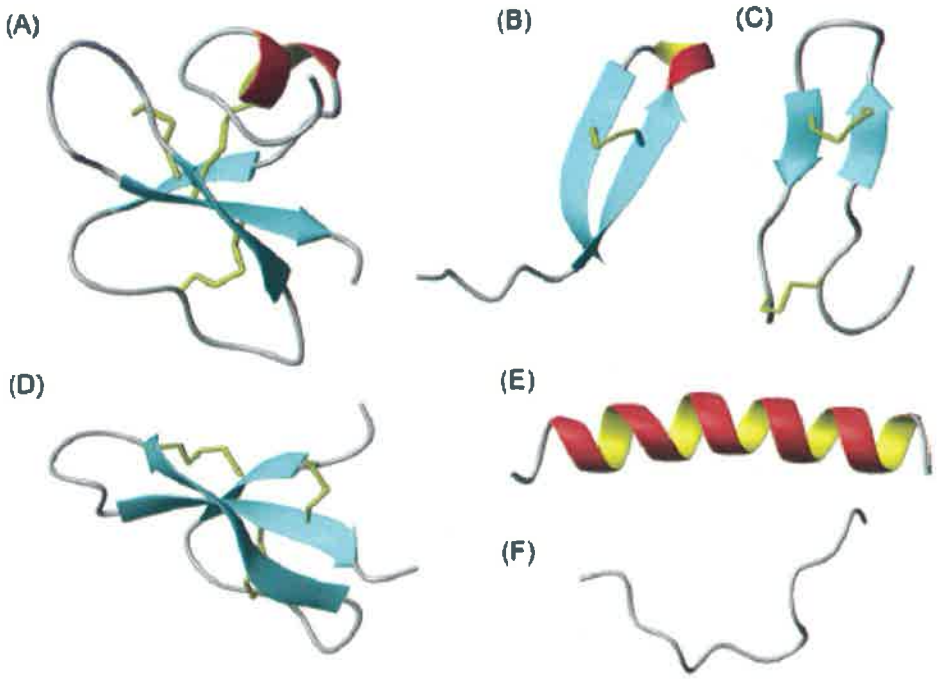


Figure 1.2: (A) human β–defensin-2; (B) looped thanatin; (C) β–sheeted polyphemusin; (D) rabbit kidney defensin-1; (E) α-helical magainin-2; (F) extended indolicidin. The disulfide bonds are indicated in yellow. From Jenssen, H.; Hamill, P.; Hancock, R. E. W. Clinical Microbiology Reviews 2006, 19, 491-+

Only a few typical secondary structures can be distinguished among the great diversity of HDPs. Some linear peptides, for example, can fold into an amphipathic alpha-helix, although many of the representative sequences, such as magainin 2, adopt this conformation only when in contact with a cell membrane or membrane mimetic and remain otherwise in a random conformation (Figure 1.2 (E)). A potential correlation between the alpha-helix content of a sequence and its propensity to induce eukaryotic cell haemolysis has been proposed[41c, 46, 48].

1.5.4 Cationic Host Defence Peptides as anticancer agents

There is clearly an urgent need to develop new approaches to cancer therapy that have a higher degree of selectivity for neoplastic cells as well as avoiding the problem of chemoresistance. Cationic host defence peptides are more commonly known as cationic antimicrobial peptides (CAPs) and show an exciting potential as a

new class of anticancer agents. The cytotoxic effect of CAPs on microorganisms and neoplastic cells is largely believed to be a function of their cationic nature and secondary structure as described above[49]. CAPs offer several important advantages over the chemotherapy drugs being used at the moment for the treatment of human malignancies. At the concentrations that it takes for peptides to kill cancer cells many CAPs do not show cytotoxic activity against untransformed proliferating cells[50]. This suggests that the CAPs can be administered *in vivo* with minimal nonspecific toxicity. However it is worth nothing that screening should be in place for haemolytic activity as CAPs at high dose concentrations have known to cause red blood cells to lyse[51]. It has also been predicted that dormant or slow-growing cancer cells with chemo-resistance will be sensitive to CAPs that kill by direct membrane lysis of the cell, as this cytolytic process does not depend on the proliferating status of the target and should bypass most multidrug resistance mechanisms[17]. Although they are known for their antibiotic activity, it has also been shown that CAPs can kill non-dividing cells[52]. CAPs have also the ability to interfere with tumor associated angiogenesis, although CAPs have been found to promote blood vessel development. CAPs with antiangiogenic activity that are also directly cytotoxic for human cancer cells have the potential to attack solid tumors through 2 independent and complementary mechanisms. CAPs may also be involved indirectly in interfering with tumor growth via their ability to modulate the host immune response[53]. Table 1.2 represents the amino acid sequences of cationic antimicrobial peptides with anticancer activity.

Table 1.2 Amino acid sequence of 'popular' cationic antimicrobial peptides with anticancer activity

Peptide	Amino acid sequence
BMAP-28	GGL**R**SLG**RK**IL**R**AW**KK**YGPIIVPII**R**I
Cecropin A	**K**W**K**LF**KK**IE**K**VGQNI**R**DGII**K**AGPAVAVVGQATQI**AK**
Cecropin B	**K**W**K**VF**KK**IEKMG**R**NI**R**NGIV**K**AGPAIAVLGEA**K**AL
SB-37	MP**K**W**K**VF**KK**IE**K**VG**R**NI**R**NGIV**K**AGAIAVLGEA**K**ALG
Shiva	MP**R**W**R**LF**RR**ID**R**VG**K**QI**K**QGIL**R**AGPAIALVGDA**R**AVG
Defensins	
Human neutrophil P1	ACYC**R**IPACIAGE**RR**YGTCIYQG**R**LWAFCC
Human neutrophil P2	CYC**R**IPACIAGE**RR**YGTCIYQG**R**LWAFCC
Human neutrophil P3	DCY**R**IPACIAGE**RR**YGTCITQG**R**LWAFCC
Human neutrophil P4	VCSC**R**LVFC**RR**TEL**R**VGNCLIGGVSFTYCCT**R**V
hBD-1	DHYNCVSSGGQCLYSACPIFT**K**IQGTCY**R**G**K**A**K**CC**K**
hBD-2	GIGDPVTCL**K**SGAICHPVFCP**RR**Y**K**QIGTCGLPGT**K**
LfcinB	F**K**C**RR**WQW**R**M**KK**LGAPSITCV**RR**AF
Magainin 2	GIG**K**FLHSA**KK**FG**K**AFVGEIMNS
MSI-136	GIG**K**FL**KK**A**KK**FA**K**AFV**K**MNN
MSI-238	D-GIG**K**FL**KK**A**KK**FA**K**AFV**K**MNN
Mellitin	GIGAVL**K**VLTTGLPALISWI**KRKR**QQ
P18 hybrid	**K**W**K**LF**KK**IP**K**FLHLA**KK**F
Tachyplesin	**K**WCF**R**VCY**R**GICY**RR**C**R**

Bold Text indicates amino acids residues that are positively charged at neutral pH. Figures adapted from Expert Opin. Investig. Drugs (2006) 15 (8): 933.

The large numbers of CAPs that exist in nature constitute a rich source of potential novel anticancer agents. Table 1.2 shows just some of the well documented CAPs which exhibit anticancer activity against human cancer cells.

1.5.5 CAP interactions with cancer cells

It is believed that CAPs associate with bacteria via interactions with negatively charged molecules such as lipopolysaccharide present in the outer membrane of Gram-negative bacteria. It has been proposed that the negatively charged outer membrane surface of bacteria and the higher negative trans-membrane potential ($\Delta\varphi$) accounts for the preferential binding of CAPs, which have a net positive charge[54]. For example, various bacteria in the mid-log phase have a $\Delta\varphi$ ranging in the region of -130 to -150 mV[47a]. This varies with eukaryotic cells which have a $\Delta\varphi$ from -90 to -110 mV[54]. In addition, fluidity of bilayers, dipole movement, curvature and the content of acidic phospholipids in bilayers also play a minor role in activity. The significance of these factors is more prominently seen for the activity difference amongst sub-strains of bacteria. Destabilisation of the outer bacterial membrane allows CAPs to gain access to the cytoplasmic membrane, which is subsequently disrupted by hydrophobic interactions mediated by amphipathic CAPs.

Similar to bacteria, many cancer cells carry an overall net negative charge due to their elevated expression, relative to non-transformed cells, of anionic molecules such as phosphatidylserine (PS) and *O*-glycosylated mucins in the outer membrane leaflet[55]. This net negative charge allows electrostatic interactions to occur between the CAPs and the surface of the cancer cell. This is however in contrast to zwitterionic phosphatidylcholine (PC) in the outer membrane leaflet of healthy eukaryotic cells which confers an overall neutral charge to these cells, resulting in a reduced electrostatic interaction with CAPs. Also in cancer cells, membrane fluidity is typically increased relative to normal eukaryotic cells[56]. This facilitates cancer cell membrane destabilisation by membrane-bound CAPs. Unlike non-transformed cells, cancer cells also have microvilli present on their surface. So the combination of the net negative charge and the increased overall surface area provided by microvilli enhance the activity differentials of CAPs between cancer and healthy eukaryotic cells[17].

Also, the outer mitochondrial membrane in eukaryotic cells possesses a net negative charge, resulting from the relative abundance of the anionic lipid cardiolipin, and a much higher $\Delta\varphi$[30]. Prokaryotic cells and mitochondria are believed to have developed from the same evolutionary ancestor[57]. Some CAPs gain access to the cell membrane via the cytosolic compartment of cancer cells by membrane destabilisation or toroidal pore formation. This can be seen in Figure 1.3 which illustrates the interactions with some neoplastic eukaryotic cancer cells. Once inside the cells, these CAPs may disrupt the negatively charged mitochondrial membrane. This can then result in the release of several different mitochondrial proteins that may induce the apoptosis pathway and result in cell death. Lactoferricin B and BMAP-28 are two CAPs which have been proven to trigger the apoptotic pathway in cancer cells by depolarisation of the mitochondrial membrane. However some α-helical CAPs with anticancer activity can also induce cancer cell necrosis as a result of CAP-mediated damage to the cell membrane[58]. It is worth noting that under physiological conditions, eukaryotic human cells are resistant to CAPs but there have been cases of cytotoxicity reported at high concentrations of CAPs such as LL-37 and DPI[59]. The cytotoxicity of these compounds should be assessed in terms of the limitations of the experiment in question. It is generally believed that the difference in the actions of these CAPs on the surface membrane is to do with the distribution of their amino acid residues and whether the sequence contains large hydrophobic or hydrophilic motifs.

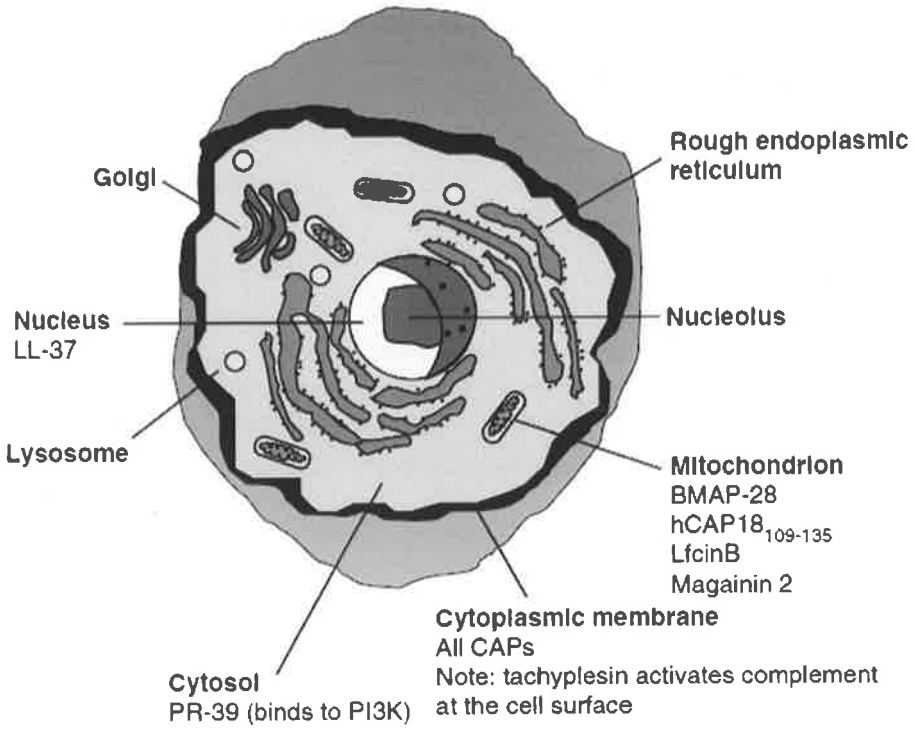


Figure 1.3: CAP interactions with neoplastic cancer cells. From Expert Opin. Investig. Drugs (2006) 15(8):933-946

1.5.5.1 Modes of action

Several models have been developed to explain the interaction of CAPs with the cell membrane that result in cell death. It is almost impossible to predict the mechanism of action of the CAPs by simple inspection of their amino acid sequence. The different modes of action are subject to controversy and there is a degree of uncertainty in the model assigned to a peptide, which also depends on the cells themselves and the concentration of the peptide. The different models described to date include the barrel-stave model, the carpet model, the toroidal or two state model, the detergent-like effect model and the in-plane-diffusion model[60].

1.5.5.1.1 Barrel and stave model (Figure 1.4)

The Barrel and stave model was first proposed by Ehrinstein and Lacar in 1977. According to this particular model, a variable number of individual peptide molecules align themselves to form a barrel like shaped pore or channel in the membrane. The peptide hydrophobic surfaces interact with the acyl chains of lipids in the membrane, generating a pore consisting of least four peptides[61,41b]. The crucial step in the model is that peptides need to recognise each other in the membrane bound state. It is highly energetically unfavourable for a single peptide to traverse the membrane, therefore the peptides line up on the surface of the membrane and aggregate on the surface until the threshold concentration is reached. It is only then that the peptide is able to insert itself into the core of the membrane by undergoing a conformational phase transition. This process forces the polar phospholipids , which make up the membrane, aside to induce localized membrane thinning[62]. This is followed by an increase in the number of peptides around and also into the channel which in turn leads to an increased pore size and stabilization. Ultimately, this causes the cell to leak its intracellular components. It is important to note that the barrel and stave method cannot account for any cytolytic activity of peptide sequences shorter than 23 residues, as these peptides do not have sufficient length to span the cell membrane [17].

1.5.5.1.2 The Toroidal Pore model (Figure 1.4)

Contrary to the pores of the barrel and stave model, toroidal pores can be formed by a greater variety of different peptides. Like the barrel and stave pore, peptides forming toroidal pores line up parallel on the surface of the membrane[63]. When the peptide threshold concentration is reached, the peptides insert themselves into the membrane or induce a positive curvature strain in the membrane resulting in an opening. This opening is known as the toroidal pore. When the peptide concentration increases, or while it is increasing, the peptide first adsorbs onto the membrane surface, aggregates and imposes thinning of the membrane and the

expansion of the head group; this induces a bending of the bilayer so that the upper and lower leaflets meet, giving the toroidal appearance[64]. The pore is usually well structured with a mixture of peptides and lipid head groups lining the interior; it's smallest in the middle and largest at the end.

1.5.5.1.3 The Carpet Model (Figure 1.4)

In the carpet model, the peptide has a carpet-like alignment between the anionic components of the phospholipids that are parallel to the cell surface. This takes place at a critical threshold concentration which will result in membrane destabilisation and disintegration. This is due to the curvature stress and osmotic pressure without the direct insertion of the peptide into the hydrophobic core of the lipid bilayer. At low concentrations the peptide magainin has been suggested to function according to the carpet model but is also believed to mediate cytolysis via the barrel stave method at higher concentrations[17].

1.5.5.1.4 The detergent like model

In the detergent like model, membrane disruption is caused by the release of micellar structures which is determined as membrane bleeding. This happens in areas of high peptide concentration. However it must be noted that there is not much evidence to support that peptides mediate cytotoxicity by this model[17].

1.5.5.1.5 In plane diffusion model

The in plane diffusion model can account for some cytotoxicity effects of α-helical peptides at low level concentration, as well as for peptides that are too short to span the cell membrane. In this particular model, peptides insert themselves in the membrane and disturb the packing of the membrane bilayer. Models such as these, which best describe the cytotoxic peptide membrane interactions, may also allow one to predict the effectiveness of individual peptides as anticancer agents[17].

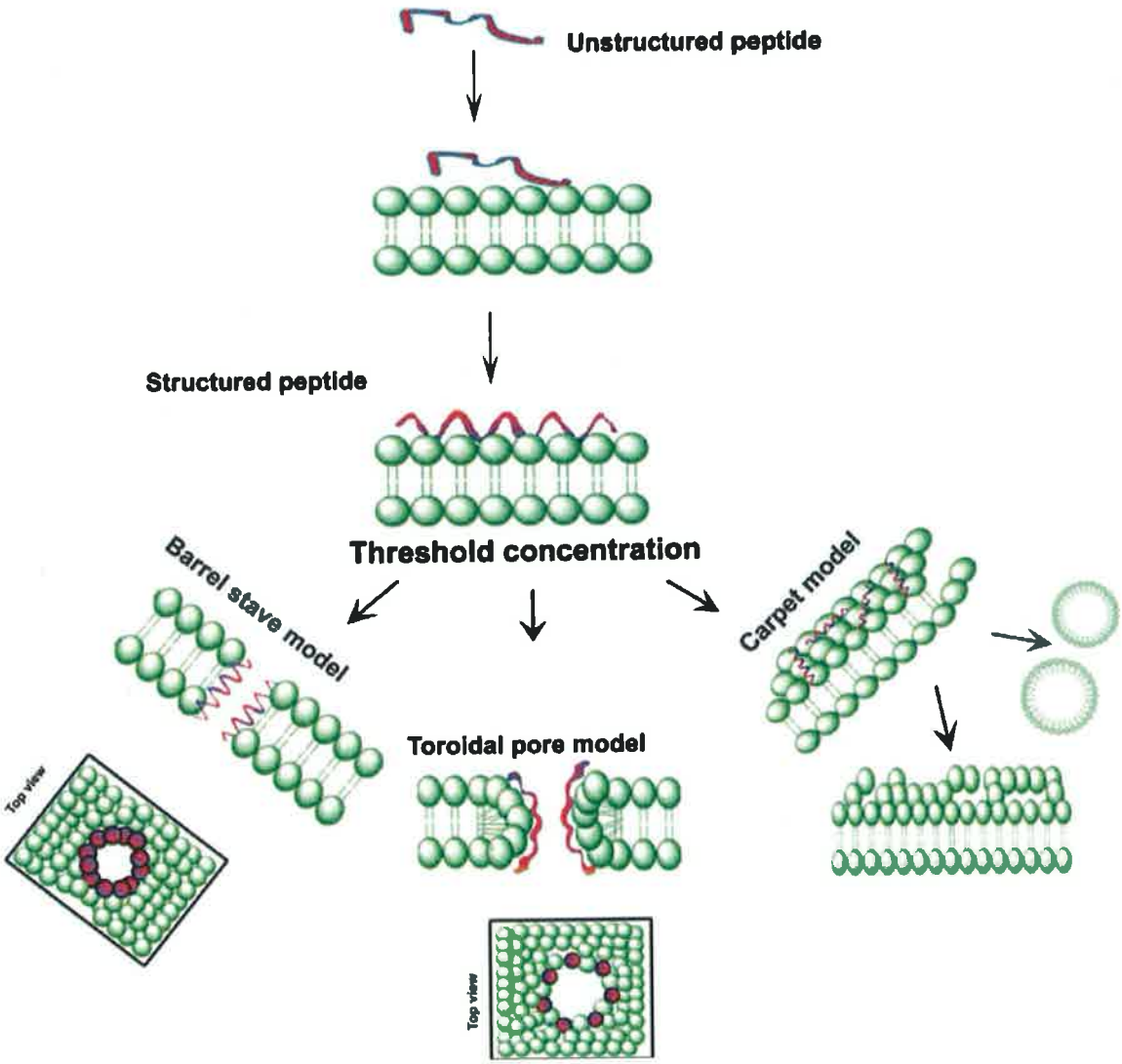


Figure 1.4: CAPs and their different modes of action. From Critical Reviews in Biotechnology, 2011, 1–29, Early Online

1.5.6 α-Helical CAPs with anticancer activity

1.5.6.1 Cathelicidins

BMAP-28 is an α-helical CAP that is a member of the cathelicidin family. Within this family of CAPs other peptides include human LL-37, CAP (hCAP)-18 and porcine PR-39. BMAP-28 has being found to have cytotoxic activity against a number of human cancer cell lines including leukaemia cell lines, U937 myelogenous and also multi-drug resistant lymphoblastic cell line CEM-VLB[50]. BMAP-28 used against leukaemia

cells can cause membrane permeabilisation and the influx of Ca^{2+} into their cytosol. While membrane damage caused by BMAP-28 can induce necrosis, there is some evidence to suggest that inter-nucleosomal DNA fragmentation is observed when there is a longer exposure of leukaemia cancer cells to BMAP-28. In K562 chronic myelogenous leukaemia cells, BMAP-28 can destabilize the mitochondrial cell membrane and trigger a release of cytochrome c[65]. Within the cytosolic compartment, cytochrome c is able to promote the activation of caspase 9 and induce apoptosis[66]. However at the concentration needed for cytotoxic activity against leukaemia cells (1.5-6μM), BMAP-28 is also active against normal human lymphocytes[65].

Other Cathelicidin family members also show potential as anticancer agents. hCAP-$18_{109-135}$) which is a 27 amino acid peptide derived from the C-terminus of the α-helical hCAP-18, has recently been shown to promote apoptosis independent of the caspase pathway in SAS-H1 human oral squamous carcinoma cells, without affecting human gingival fibroblasts or keratinocytes. hCAP-18109-135 also causes a mitochondrial membrane disruption via the same mechanism as BMAP-28[65]. LL-37, a 37 amino acid peptide, also released from hCAP-18, is also taken up into mammalian cells. It is then localised within the nuclear compartment of the cells, without any signs of growth inhibition or cytotoxic effects[67]. The significance of LL-37 uptake and nuclear localisation in terms of possible anticancer activity is not yet known.

Unlike the former α-helical peptides, porcine PR-39 is a linear, proline-rich peptide that also belongs to the cathelicidin family. Human hepatocellular carcinoma cells engineered to express porcine PR-39 have an altered actin structure and reduced invasive activity, suggesting gene therapy with PR-39 as a possible approach to inhibit the growth of cancer cells. Gene transfection of PR-39 stops the growth of ras-transformed cells via the binding of PR-39 to the PI3Kp85α subunit and this in turn inhibits the PI3K activity. However PR-39 is also taken up in eukaryotic cells following its interaction with the cell membrane.

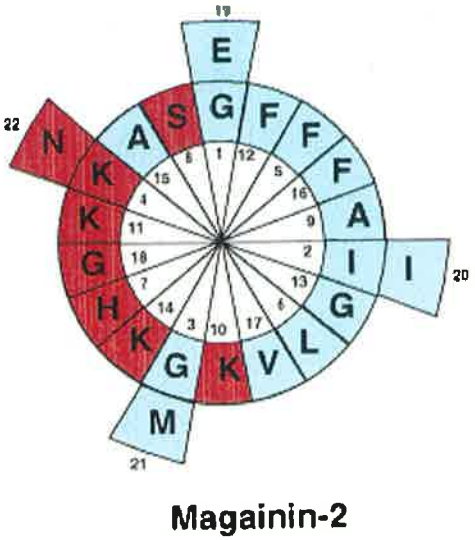
1.5.6.1.1 Cecropins

Cecropins are a group of CAPs that contain approximately 34-39 amino acid residues and are present in mammals as well as insects, including the giant silk moth *Hyalophora cecropia*[68]. They are subdivided into two different families of peptides, known as cecropin A and cecropin B. All of the cecropin peptides show sequence homology and adapt an α-helical, amphipathic structure with a N-terminus consisting of mainly basic amino acid residues and a hydrophobic C terminus[69]. This group of peptides display anticancer activities against many different types of cancer cell lines including leukaemia and lymphoma cells. In addition, at the concentration that it takes to kill transformed cancer cells, cecropins are unable to lyse normal fibroblasts, lymphocytes or mammalian erythrocytes[70]. They are also found to be active against multidrug resistant breast and ovarian cancer cell lines. It has been found that combination studies between cecropin A and chemotherapeutic agents such as 5-fluorouracil and cytarabine have proven to have supra-additive effects against CCRF-SB lymphoblastic cancer leukaemia cells. This suggests that other α-helical CAPs in combination with classical anticancer drugs is a potential therapeutic approach still left to be fully investigated[17].

1.5.6.1.2 Magainin 2

Magainin 2 is an α-helical peptide which consists of 23 amino acids and is derived from an African clawed frog *Xenopus laevis*[71]. Magainin displays selective cytotoxicity against cancer cells and is found to be active against transformed cells at concentrations levels <5-10 times lower than those necessary to kill untransformed human cells[72]. Magainin analogues, magainin A and magainin G, have also been developed with much improved α-helical composition and also reduced haemolytic activity in comparison to the original native magainin 2 (Figure 1.5) [73]. These two analogues have been found to have activity against human small cell lung cancer lines and also to be able to permeabilise and penetrate the cell membrane of HeLa human cervical carcinoma cells in an energy- and receptor-

independent manner[17]. Studies have shown that magainins are capable of interacting with cancer mitochondrial membranes and are able to trigger apoptosis by release of cytochrome c with subsequent activation of caspase-9[66]. *In vivo* studies of magainins anticancer activities shows their potential as therapeutic agents due to their resistance to degradation, decrease in tumour cell invasion into soft tissues and the overall survival rate within the animal studies.



Magainin-2

Figure 1.5: Schiffer-Edmundson wheel projection of the Magainin 2 sequence. From Biopolymers, Peptide science, Vol. 47, 451-463

1.5.6.1.3 Melittin

Melittin is an amphipathic α-helical CAP made up of 26 amino acid residues isolated from the venom of the common bee *Apis mellifera*[74]. Mellitin has the ability to kill ras-transformed cells by a mechanism that involves the hyper activation of cellular phospholipase A_2 through a melittin-mediated influx of Ca^{2+} [75]. When ras-transformed fibroblast cells, NIH3T3, are repeatedly exposed to melittin, reduction of ras-oncogene expression is observed. However, a population of cells with reduced ras expression also display resistance to melittin. This may undermine the therapeutic potential of melittin as an anticancer agent. Nevertheless, melittin based therapeutic agents still attract interest. Melittin-avidin is a melittin conjugate

based on a matrix metalloprotease-2 (MMP-2) linker. This enzyme, whose expression is increased in cancer cells, is able to cleave the linker and release melittin from avidin at the site of the tumour cell[76]. While the intact mellitin-avidin conjugate is inactive, upon cleavage it is a potent killer of cancer cells which express MMP-2, including SKV03 ovarian cancer cells and DU 145 prostate cancer cells. When used against solid tumours in mice models, mellitin-avidin induced a significant reduction in tumour size. The selective targeting of a CAP through the over-expression of MMP-2 in cancer cell lines is a promising strategy for peptide-based anticancer therapeutic agents[17].

1.5.7 β-Sheet CAPs with anticancer activity

1.5.7.1 Defensins

Defensins are a group of cysteine- and arginine-rich CAPs that have been isolated from a number of different species including molluscs, insects and mammals[30]. Mammalian α- and β-defensins contain a β-sheet structure that is stabilised by up to 3 disulphide bridges. Human neutrophil peptide (HNP) defensins make up 4 of the 6 types of α-defensins and the other two types are found in Paneth cells. The human β-defensins are expressed in epithelial cells of many different organs and are named hBD-1 to hBD-6[77].Defensins carry a very broad spectrum of activity, mostly identified as antimicrobial, however they also show potential for the treatment of other diseases (Figure 1.6). This includes cancer, through their ability to kill a variety of human and murine tumour cell lines. For example, HNPs 1 to 3 defensins can cause lysis of IM-9 myeloma cells and Raji B lymphoma cells within 3 hours of exposure to these CAPs[78]. There may be some drawbacks to the use of defensins as anticancer agents, as in low concentration they have been shown to promote growth in untransformed cells such as fibroblast and epithelial cells via the activation of MAPKs. In higher concentrations they have been shown to be cytotoxic to all cell types and can cause undesired side-effects.

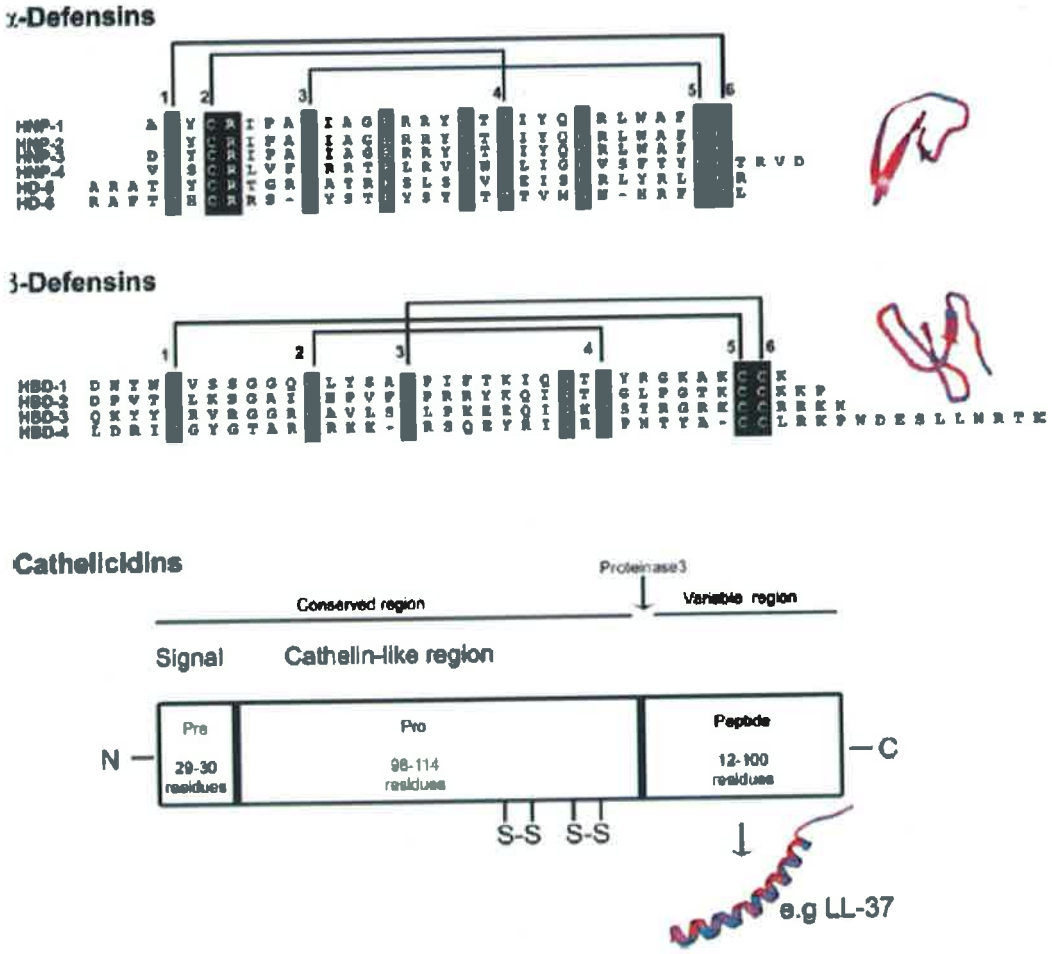


Figure 1.6: Sequence homology of Defensins and Cathelicidins: From Critical Reviews in Biotechnology, 2011, 1–29, Early Online

1.5.7.1.1 Lactoferricin

Lactoferricin is an amphipathic CAP produced by acid-pepsin hydrolysis of lactoferrin[79]. Lactoferrin is an 80kDa iron building glycoprotein in the exocrine secretions of saliva, milk and also in the secretory granules of neutrophils. Bovine LfcinB (Lactoferricin B), with the use of NMR spectroscopy has shown to adopt an amphipathic structure, with the hydrophobic amino acids found on one face and the remaining positively charged amino acid residues on the other face. Beside its antimicrobial activity, *in vitro* studies have shown that LfcinB has also the ability to kill a broad spectrum of human tumour cell lines which include sarcoma, carcinoma, leukaemia and neuroblastic cells[80]. In addition, LfcinB doesn't harm untransformed cells including fibroblast cells, T lymphocytes and epithelial cells at concentrations

that are harmful to cancer cells. *In vivo* LfcinB is also a very potent anticancer agent with activity which prevents tumour growth and metastasis in many different types of mouse models. LfcinB also possesses antiangiogenic activity, inhibiting the development of tumour associated vasculature in mice bearing syngeneic B16-BL6 melanoma cells[81]. The selective cytotoxic activity of LfcinB against neoplastic cells suggests that there should be little or no nonspecific toxicity associated with its use as an anticancer agent.

1.5.7.1.2 Tachyplesin

Tachyplesin is a 17 amino acid CAP which is amphipathic in structure and is comprised of two beta sheets held together by two disulphide bonds. Tachyplesin was isolated from the haemocytes of the horseshoe crab *Tachypleus tridentatus*[82]. Due to its secondary structure, tachyplesin adopts a conformation where all the basic amino acids are exposed on its surface. It has been shown that the activity of tachyplesin against different types of cancer cells relies on different mechanisms. In the human hepatocarinoma cancer cell line SMMC-77721, tachyplesin inhibits the cell proliferation but increases the expression of tumour suppressor gene $p21^{WAF1/CIPI}$ and decreases expression of the *c-myc* oncogene. This is in contrast to a synthetic fusogenic sequence of tachyplesin and of an integrin homing domain sequence arginine-glycine-aspartic acid, (RGD) which triggers caspase-dependent apoptosis in culture of human TSU prostate cancer and endothelial cells[83]. It also inhibits the growth of TSU prostate cancer xenografts on chorioallant membranes of chicken embryos as well as growth of B16 melanoma cells in syngeneic mice. Human serum proteins promote rather than interfere with growth inhibitory effects of tachyplesin on prostate cancer cells, because the complement cascade is not the only mechanism by which tachyplesin affects prostate cancer cells. Indeed, heat inactivation of serum to destroy complement proteins attenuates (but does not eliminate) the inhibitory effect of tachyplesin on TSU prostate cancer growth[84]. Being able to kill cancer cells in the presence of high levels of serum is an important

feature in terms of the possible therapeutic utility of tachyplesin, although the nonspecific cytotoxic effect of tachyplesin on endothelial cells may be problematic.

1.5.7.1.3 Hybrid CAPs and the introduction of P18

Recent studies have shown that combining different sequences of antimicrobial peptides in the same synthetic peptide can generate more effective sequences than the parent peptides. P18 is a hybrid cationic peptide derived from cecropin A and Magainin 2[85]. P18 is formed by combining the positively charged N-terminus of the α-helix of cecropin A (1-8) with the C-terminus amphipathic α-helix of magainin 2 (1-12). It was found to possess potent cytotoxic activity against bacterial and cancer cells, without displaying any significant haemolytic activity[86]. A proline hinge in the P18 peptide has been shown to enhance the cytotoxicity of the peptide against cancer cells. If the proline is replaced by a serine or leucine residue, there is a decrease in the cytotoxic activity against cancer cells but an increase in the haemolytic activity. This indicates that the primary structure is a determinant of P18 selectivity for neoplastic cells. A similar anticancer peptide is the hybrid sequence of cecropin A-mellitin. These hybrid CAPs selectively kill a number of human cancer cells and are attractive candidates as novel anticancer agents.

1.5.7.2 Conclusion

Peptides of the innate immune system have been shown to be exciting therapeutic candidates due to their ability to avoid chemoresistance and to target cancer cells. They can potentially be selective against bacterial and cancer cells at low concentrations that are not harmful to normal human cells. However, the drawbacks of peptides as anticancer agents are their low activity as single agents, susceptibility to enzymatic degradation and risk of potential immunogenicity. There are approaches to improve the efficiency of these anticancer peptides including conjugation of small drug molecules or of monoclonal antibodies. This may allow targeted delivery to cancer cells and minimise the effects of cancer therapeutics

against normal tissues. Although a lot of these therapies have been shown to be attractive anticancer agents, they fail to address the problem of enzymatic degradation and inactivation of positively charged anticancer peptides by anionic serum components. Targeted liposomal delivery vehicles have been shown to deliver anticancer peptides to tumour sites and simultaneously to protect peptides from enzymatic degradation and inactivation by serum proteins, as well as reducing the immunogenicity of these foreign anticancer peptides. Liposomes are small spherical vesicles composed of a membrane phospholipid and cholesterol bilayer that allows for the encapsulation and delivery of drugs, proteins or other therapeutic agents via the intravenous route with enhanced cargo activity and diminished toxic side-effects[17]. Synthetic polymers represent also another delivery tool for peptide delivery. A lot of these techniques can be used in the form of prodrugs.

1.6 Prodrugs

Prodrugs are derivatives of drugs which ideally remain inactive until they are specifically metabolised or activated at one or more target body site(s). They are particularly useful in the development of novel anti-tumour chemotherapeutic drugs based on cytotoxic agents, leading to reduced side-effects associated with their toxicity, improved specificity and in some cases the avoidance of multidrug resistance mechanisms. The use of anticancer prodrugs for targeted therapy is usually based on tumour-associated cell surface markers, such as antigens or receptors, whose expression differs between normal and cancer cells. A schematic representation of the prodrug principle can be seen below.

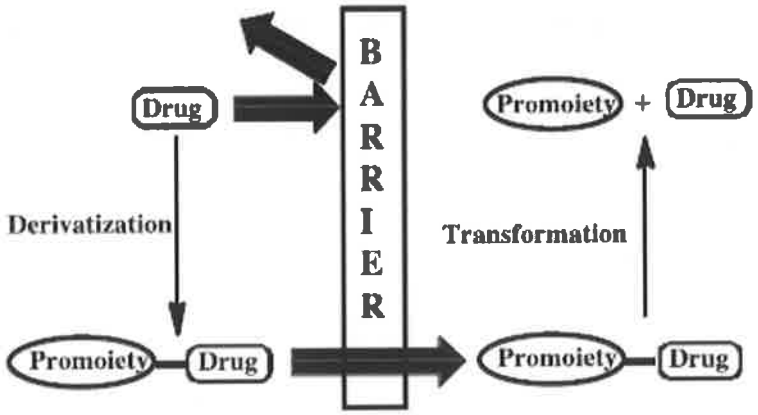


Figure 1.7: Schematic Representation of prodrugs concept taken form Prodrugs: Challenges and rewards, AAPS Press, Springer,

The "Barrier" in this scheme represents the problem(s) preventing effective drug delivery. There could be a range of different problems which the barrier might represent including; solubility, bioavailability, toxicity, immunogenicity, circulatory retention time and degradation. The promoiety represents the mask which temporarily allows the drug to overcome these barriers but is later removed to re-establish the drug's activity (Figure 1.7)[87]. In order to develop a prodrug certain criteria have to be met. The ideal drug is one that is active, easy to formulate, well absorbed after oral dosing, has an acceptable phararmacokinetic profile, and is both renally cleared and metabolized to 1–2 non-toxic metabolites that are rapidly excreted after being formed. The ideal prodrug, therefore, is one that readily achieves its desired goal, is non-toxic, and breaks down efficiently and quantitatively to the drug and to known and safe by products. Like the drug discovery process, this goal is not often met. In order to resolve the pharmacokinetic and toxicological properties of drug candidates remains a key challenge in drug development. The aim is to be able to design a drug that has an efficient permeability to be absorbed into the blood circulation and to be stable against metabolism and also avoid being eliminated from the body[88]. For the ideal pharmacokinetics, the must display the following strategies (1.6.1.1 - 1.6.1.4);

1.6.1.1 Improved absorption

In order for drug absorption to take place it has to have the right balance between hydrophobic and hydrophilic properties. If the compound was too polar, it would not be able to pass the cell membrane of the gastrointestinal (GI) barrier. It would have to be injected intravenously into the body and then face a problem of being rapidly eliminated from the body[89]. On the other hand, non-polar drugs would be poorly absorbed through the membrane. If they were given by injection, they would be more than likely retained in fat tissue. Therefore the hydrophobic and hydrophilic balance is crucial in the design of a prodrug.

1.6.1.2 Improved metabolism

Improving metabolism can involve utilizing a number of different strategies. For example, adding a steric shield to some functional groups that may be more susceptible to hydrolysis than others or that the drug could be subject to enzyme degradation.[90] Having some sort of protection group mechanism in place is another crucial piece of the prodrug design.

1.6.1.3 Reduced toxicity

It is often found that in drug trials, that the main reason for a drug failing is directly related to the toxicity. This may be due to toxic metabolites, in which case the drug should be more resistant to metabolism. It is known that functional groups such as aromatic nitro groups, aromatic amines, bromoarenes, hydrazines, hydroxylamines or polyhalogenated groups are generally metabolized to toxic metabolites. To reduce the toxicity of these substances an approach to reduce or eliminate these substituents from the active drug is needed. However, these functional groups can also be important to increase cytotoxic effects depending on the design of the drug in question.

1.6.1.4 Targeted prodrugs by metabolic enzymes

The principle of targeting drugs can be traced back to Paul Ehrlich who developed antimicrobial drugs that were selectively toxic for microbial cells over human cells. This method for achieving this is to design drugs which make use of specific molecular transport systems[91]. This approach may be utilized in the cases of where the parent drug faces problems associated with solubility, instability, toxicity or formulation. A metabolic enzyme is usually involved in converting the prodrug to their active forms. It should be noted that not all prodrugs are activated by metabolic enzymes but metabolic enzymes are available in the cell already and can be very efficient at cleaving the prodrug[92].

In most cases prodrugs contain a promoiety (linker) joined to the parent drug molecule. These linkers are usually different and are cleaved by different mechanisms. In some cases, two biologically active drugs can be linked together in a single molecule called a codrug. In a codrug, each drug can act as a linker for each other[93]. The prodrug approach has been used to overcome various undesirable drug properties and to optimize clinical drug application. The exploitations of different proteins and markers discovered through advances in molecular biology have provided direct availability of enzymes and carrier proteins along with knowledge of their molecular and functional characteristics. A targeted drug design approach by considering enzyme-substrate specificity or carrier substrate specificity in order to overcome various undesirable drug properties is therefore important. This type of targeted drug design requires extensive knowledge of particular enzymes or carriers and all their molecular functions.

Chapter 2:

Polymeric Peptide Prodrugs

2 Introduction

2.1 Overview

Chemotherapy combinations have been the mainstays in the treatment of most types of cancer in the past half century[17]. Multi-drug therapies may circumvent mechanisms of tumour resistance to single agents and potentially provide greater therapeutic benefits if the selected agents act synergistically in combination. A multi-component drug delivery system combining a classical cytotoxic drug, doxorubicin (DOX), and a host defence peptide (P18) was therefore developed by conjugating these 2 candidates to a polymeric carrier through a reversible linkage (Figure 2.1). A polymer of ethylene glycol (PEG) was chosen as the carrier because of its non-toxic and non-immunogenic properties[94]. PEGylation of a peptide can also improve its pharmacokinetic and pharmacodynamic properties and enhance its accumulation in tumour tissue by the "enhanced permeability and retention (EPR) effect"[95].

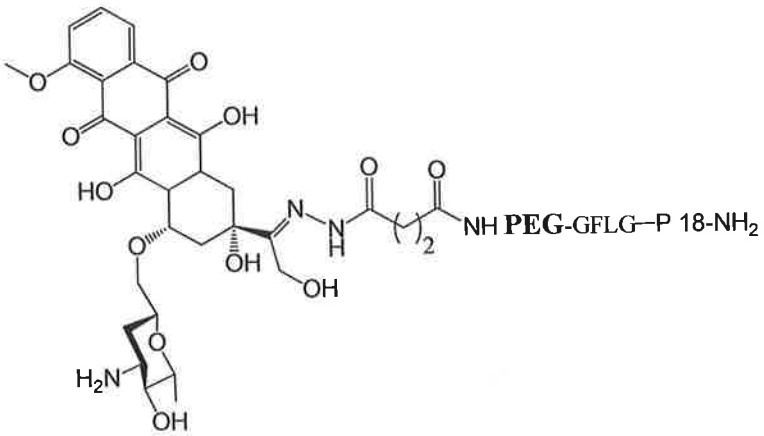


Figure 2.1: A multi component drug delivery system combining a classical cytotoxic drug, doxorubicin (DOX), and a host defence peptide (P18) to a polymeric carrier[2]

A method for the reversible PEGylation of P18 (H-kwkflkklpflkhalkkf-NH_2)[96], a hybrid sequence of cecropin A (1-8) and magainin 2 (1–12) with known anticancer activity[85] and its conjugation with a classical anticancer agent was originally developed. In this approach, which generates a polymeric dual-release prodrug of the peptide and the conventional agent, the P18 sequence assembled from D-amino acids was elongated at its N-terminus by a short peptide linker Gly-L-Phe-L-Leu-Gly. This GFLG sequence is a substrate of the endo-lysosomal cathepsin B enzyme, a protease highly up-regulated in wide variety of cancer cells[97]. The N-terminal amino group of this linker was in turn aminated with a bi-functional PEG (H_2N-PEG-COOH) in which the amino group was modified with a hydrazide linker for conjugation to doxorubicin via a hydrazone bond. The latter is stable at neutral pH, but can be hydrolysed in the lysosome at pH lower than 4.5[98]. PEGylation of doxorubicin can be used to prevent its toxicity and target its delivery to malignant cells by the EPR effect. Following passive accumulation in the tumor and cellular uptake by endocytosis, it is proposed that release of the anticancer peptide and classical agent could occur in the lysosome of cancer cells, thereby allowing their combined and selective delivery.

While the synthetic feasibility study for the production of these conjugates was successfully completed with the details and characterization of these found in this section, the linear multi-step synthetic strategy implemented was not amenable to convenient scale-up. Furthermore, the design of these polymeric dual-release prodrugs limits their application to a 1:1 doxorubicin-peptide ratio, which might not be adequate due to the significant difference in the anticancer activities of these 2 candidates (nanomolar range for doxorubicin, micromolar range for P18). Therefore, an alternative approach based on the combination of individual polymeric prodrugs of P18 and doxorubicin was developed to study their potential synergistic/additive effects.

2.2 PEGylation

2.2.1 Source

In the late 1960s, it was first noted by Frank Davis *et al.* that attaching a hydrophobic polymer, for example a fragment of dextran or glycogen, or some sort of biopolymer, to a protein might reduce its immunogenicity. From this observation Davis concluded that polymers could have medical applications. Until then polymers were regarded mainly as industrial or commercial agents. From his research in this new application, one polymer became the focus of Davis' research, polyethylene glycol (PEG), because of its hydrophilic and non-toxic nature. Davis and his group focused therefore on synthesising PEG-proteins. During their first studies with low molecular weight PEGs, they noticed that PEGylated proteins had not only greatly reduced immunogenicity *in vivo* but also had much improved circulating lives than their unPEGylated counterparts. From here a new era of PEGnology was born[99].

PEG has become a highly investigated polymer for the covalent modification of biological macromolecules and surfaces in many pharmaceutical and biotechnical applications. Within these biological macromolecules, peptides and proteins are of extreme importance. Reasons for PEGylation, the covalent attachment of PEG, to peptides and proteins, are numerous. They include shielding of antigenic and immunogenic epitopes, shielding receptor-mediated uptake by the reticuloendothelial system (RES) and preventing recognition and degradation by proteolytic enzymes. PEG conjugation also increases the apparent size of the polypeptide, thus reducing renal filtration and altering biodistribution. An important aspect of PEGylation is the incorporation of various PEG functional groups that are used to attach the PEG to the peptide or protein[100]. (Figure 2.2)

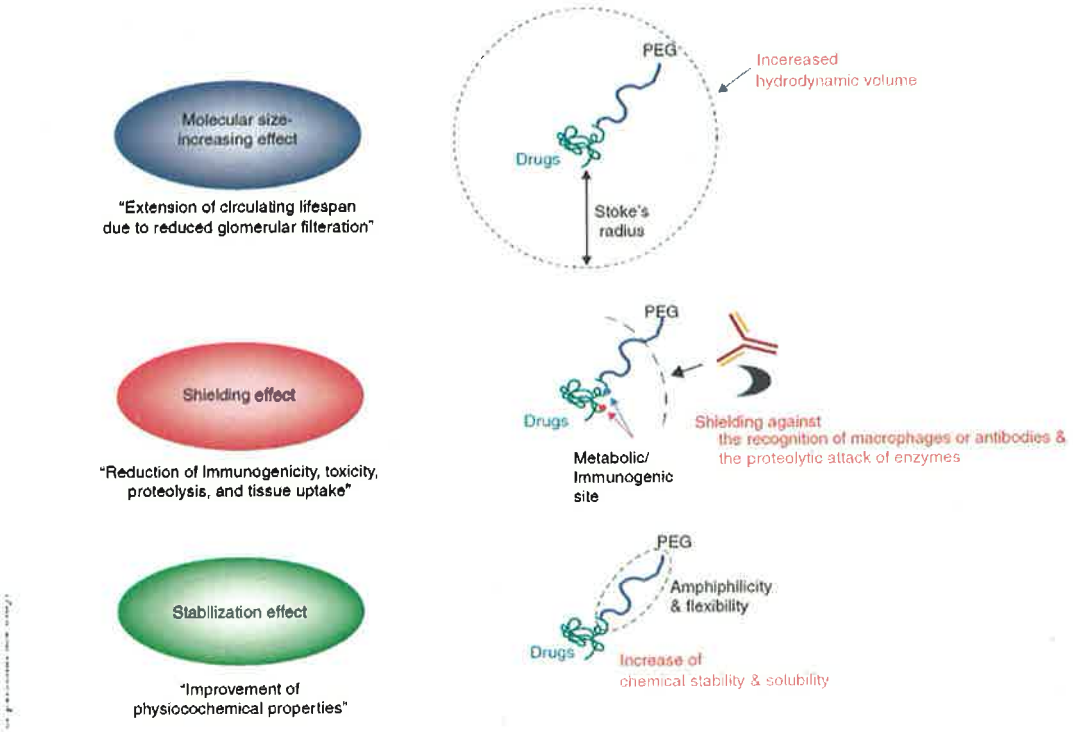


Figure 2.2: The molecular size increasing, shielding and stabilizing effect of PEG. From Expert Opin. Emerging drugs (2009) **14**(2) 363.

The use of peptides and proteins has expanded in recent years due to the discovery of novel peptides and proteins with potential applications as human therapeutics. There has also been a better understanding in their mechanisms of action *in vivo* and also improvements in their expression and synthesis. Advances in formulation or molecular-altering technologies have also improved the delivery of these polypeptides with much improved pharmacokinetic and pharmacodynamic properties *in vivo*. It was estimated that in the year 2000, peptide and protein drugs made up about 10-35% of the biopharmaceutical drugs in clinical trials[101].

Although the growth rate of biopharmaceutical drugs is nearly double the growth rate of low molecular weight agents for APIs overall, they do not come without their problems. Polypeptide therapeutics have to overcome problems associated with short circulating half-life,[102] immunogenicity, proteolytic degradation and in some cases poor solubility. Several different approaches have evolved to enhance the pharmacokinetic and pharmacodynamic properties of polypeptides. These

approaches include the manipulation of the amino acid sequences to decrease their immunogenicity and proteolytic cleavage, *e.g.* substitution of L-amino acids with D-amino acids, fusion or conjugation of immunoglobins and serum proteins (albumin),[103] incorporation into drug delivery vehicles, to protect the polypeptide or provide a slow release mechanism, and attachment to natural and synthetic polymers.

The covalent attachment of PEG to polypeptides has become quite familiar amongst those in the biomedical, biotechnology and pharmaceutical communities for reasons described previously. There are four main factors to consider in the PEGylation of peptides and proteins, the number of PEG chains attached to the sequence, the molecular weight and structure of the PEG chains, the location of the PEG chain on the backbone of the polypeptide and the chemistry used to attach the PEG to the peptide[104].

In the past few decades more PEGylated peptides have reached the market as pharmaceuticals with a lot more reaching the late stages of clinical trials. Some of the first PEGylated proteins developed and now currently on the market are Adagen®, Oncospar® and PEG-intron ® (see Table 2.1 for complete list). These drugs were all developed from the first generation of PEG chemistry. First generation PEGylated protein drugs consisted of low molecular weight linear PEGs (<12 KDa). These polymers were susceptible to side-reactions or weak linkages upon conjugation to the peptide/protein. The second generation of PEGylated protein therapeutics dealt with the problems faced in the first generation PEG chemistry and introduced polymers with a smart design with improved selectivity for conjugation and able to generate better drug candidates.

Table 2.1: Marketed PEGylated compounds (Figures from Expert Opin. Emerging drugs (2009) **14**(2) 363)

Table 1. Marketed PEGylated drugs

Brand Name	Peg Conjugates	Company	PEGylation	Approved year
Pegasys®	Peginterferon alpha-2a	Hoffman-La Roche	Random, branched 40kDa with two 20kDa linear PEG's, amine PEGylation	2002(US,EU)
Neulasta®	Pegfilgrastim	Amgen	Selective, Linear 20kDa PEG N-terminus PEGylation	2002 (US) 2003 (EU)
Somavert®	pegvisomant	Pharmacia & Upjohn	Random, 4-6 linear, 5kDa PEG's, amine PEGylation	2002 (EU) 2003 (US)
Macugen®	Pegaptanib sodium	OSI/Pfizer	Selective, branched 40kDa with two 20kDa linear PEG's, amine PEGylation	
Adagen®	Pegademase bovine	Enzon	Random, multiple linear 5kDa PEG's amine PEGylation	1990 (US)
Oncaspar®	Pegaspargínase	Enzon	Random, multiple linear 5kDa PEG's amine PEGylation	1994 (US,EU)
PegIntron®	Pegínterferon	Schering-Plough	Random, Linear 12kDa PEG, amine PEGylation	2000 (EU) 2000 (US)
Mircera®	mPEG-epoetin beta; CERA	Roche	Random, Linear 30kDa PEG, amine PEGylation	2007 (US,EU)
Cimzia®	Certolizumab pegol	UCB	Selective, branched 40kDa thiol pegylation	2008 (EU)

2.2.2 Properties of PEG

2.2.2.1 Structure of PEG

In its most common form, PEG is found to be in a linear or branched form with a polyether chain terminated by hydroxyl groups. The general structure of a linear PEG can be seen in Figure 2.3.

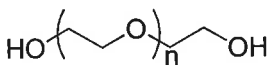


Figure 2.3: Structure of polyethylene glycol

The synthesis of PEG is performed by anionic ring opening polymerization of ethylene oxide initiated by nucleophilic attack of a hydroxide ion on the epoxide ring. The most useful PEG for polypeptide modification is the monomethoxy PEG (mPEG) with a methoxy group at one end of the PEG chain and a hydroxyl group at the other end as shown

Figure 2.4.

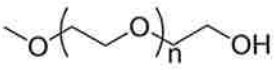


Figure 2.4: Structure of monomethoxy PEG

The synthesis of this monofunctional PEG is performed by anionic ring opening polymerization initiated with methoxide ions. Due to trace amounts of water during the polymerization step, commercially available mPEG contains sizeable amounts of diol PEG, up to as much as 15% of the composition of PEG[105].

2.2.2.2 Polydispersity of PEG

PEG has a very narrow polydispersity compared with other polymers. Polydispersity refers to the formation of an object (PEG-polymer) that has an inconsistent size, shape and mass distribution. This narrow polydispersity makes PEG attractive for PEGylation and purification. Polydispersity is measured by the polydispersity index (PDI)[106]. The PDI is a measure of the distribution of molecular mass in a given polymer sample. The PDI calculated is the weight average molecular weight (M_w) divided by the number average molecular weight (M_n). It indicates the distribution of individual molecular masses in a batch of polymers. The PDI has a value equal to or greater than 1, but as the polymer chains approach uniform chain length, the PDI approaches unity of 1. The PDI from polymerization is often denoted as;

$$PDI = (M_w) / (M_n)$$

Where (M_w) is the weight average molecular weight and (M_n) is the number average molecular weight. Distributions of low and high molecular masses have more impact on (M_n) and (M_w), respectively[107]. PEG has a PDI of 1.01 for low molecular weight PEG's (< 5kDa) and a PDI of 1.1 for PEG's with a high molecular weight (>50 kDa).

2.2.2.3 Solubility of PEG

PEG has the unique ability to be both soluble in aqueous and in most organic solvents, except ethers such as diethyl ether, even though PEG has a polyether backbone. This makes the end group conjugation to biological components easy under mild physiological conditions and attractive from a synthetic point of view because the conjugates are insoluble in ether and conveniently isolated by precipitation. Studies carried out with PEG in solution have shown that the typical ethylene oxide unit of PEG binds to 2-3 water molecules. Due to the high flexibility of the backbone chain in PEG and its association with water molecules, the PEG macromolecule acts as if it was 5-10 times as large as a soluble protein of a comparable molecular weight[108]. These factors are thought to be responsible for PEG's ability to exclude proteins from cell surfaces, reduce their immunogenicity and antigenicity and prevent their degradation by mammalian cells and enzymes.

2.2.2.4 Stability of PEG

Low molecular weight oligomers of PEG <400 Da have been shown to be degraded *in vivo* by alcohol dehydrogenase to toxic metabolites. However this is not the case for PEGs > 1000 Da which are found to be non-toxic and are found commercially in foods, cosmetics and pharmaceuticals.[108] PEG is rapidly cleared *in vivo* without any structural change and the clearance of the PEG from the body is based on its molecular weight. PEG that is <20 kDa is cleared from the body in the urine. Higher molecular weight PEGs, >20 kDa, are cleared much slower but still in the urine, although they can also be found in the feces.

2.2.3 Chemistry of PEG

2.2.3.1 The development of 1st generation PEG compounds

The first generation of PEG conjugates was essentially obtained by the reaction of activated PEG chains under mild chemical reaction conditions with the alpha amino terminus of a polypeptide chain and/or the epsilon amino groups of its lysine residues. (Figure 2.5)

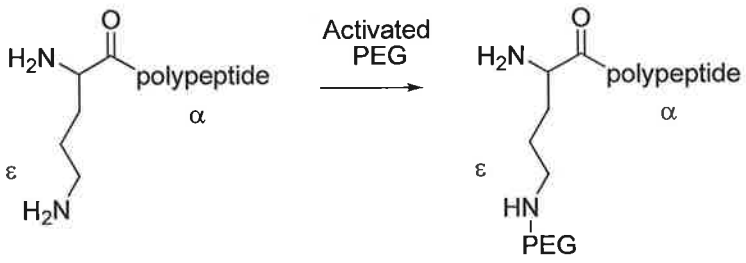


Figure 2.5: Lysine

The first generation of PEGylated proteins contained high amounts of PEG impurities and in some cases unstable linkages, was restricted to low molecular weight polymer chains and lacked selectivity in the sites for polypeptide chain

modification. Early activated PEG derivatives included Figure 2.6: (a) PEG dichlorotriazine (b) PEG tresylate. Initial PEG work by Davis *et al.* used cyanuric chloride to prepare activated PEG for the conjugation to proteins.[109](Figure 2.6 (a)).

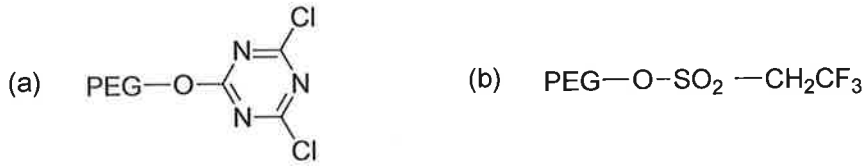


Figure 2.6: (a) PEG dichlorotriazine (b) PEG tresylate

PEG dichlorotriazine Figure 2.6 (a) can react with multiple nucleophilic functional groups such as the side-chains of lysine, serine, tyrosine, cysteine and histidine which results in displacement of one of the chlorides and produces a conjugation in the form of a secondary amine linkage[100]. The remining chloride is less susceptible to reactions with nucleophilic residues, but its reactivity is still sufficient to allow cross-linking of the protein molecule. To solve the problem of the free reacting chloride, a branched PEG molecule was developed by Indana *et al.* $mPEG_2$-chlorotriazine[110], Figure 2.7.

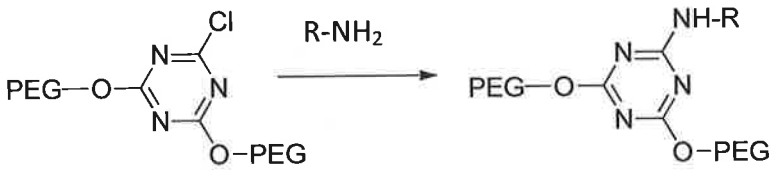


Figure 2.7: $mPEG_2$-Chlorotriazine reaction with Polypeptide (R)

The lower reactivity of the chloride enables the preferential modification of lysines and cysteines and prevents the occurrence of side-reactions at other residues. PEG tresylate, Figure 2.6 (b), is another alkylating agent that is used to modify multiple amino groups to form secondary amine linkages with proteins, viruses and liposomes. It is more specific than PEG dichlorotriazine but it has been shown by Gais *et al.* that the conjugation of small molecule amines can yield sulfamate linkages that are susceptible to degradation[111]. Besides the alkylated derivatives,

most of the first generation PEG conjugates were formed through acylation. PEG succinimidyl carbonate (SC-PEG) and benzotriazole (BTC-PEG) are widely used first generation mPEGs. They react essentially with lysines to form carbamate linkages but can also react with histidine and tyrosine residues. Other acylating reagents that can form urethane links with protein are the *p*-nitrophenyl carbonate (pNPC-PEG), trichlorophenyl carbonate (TCP-PEG) and carbonylimidazole (CDI-PEG) derivatives. These three compounds are prepared by the reaction of chloroformate or cabonylimidazole with the terminal hydroxyl group of mPEG. These compounds have a much lower reactivity than either the SC-PEG or the BTC-PEG, but it is thought that the lower the reactivity of the activated PEG the higher its specificity for certain amino acids in the protein.

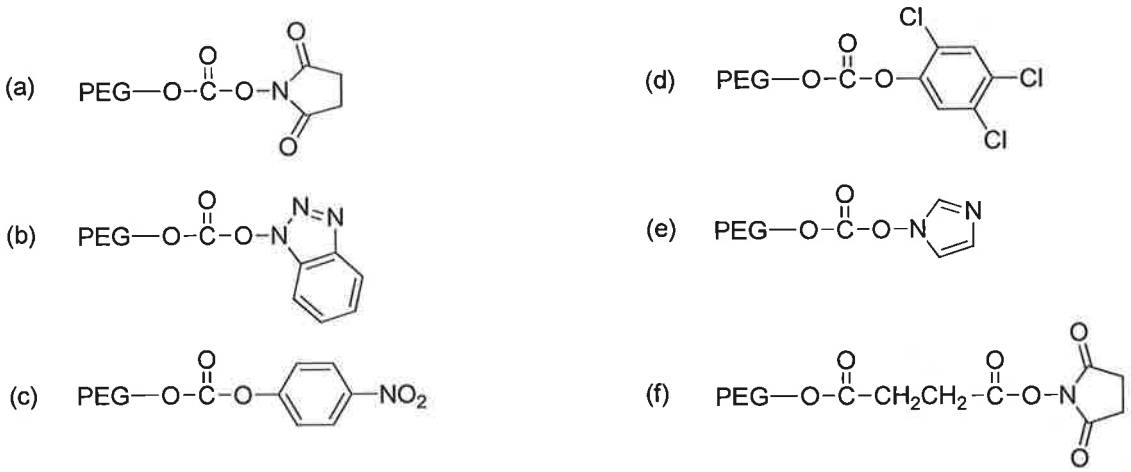


Figure 2.8: First generation PEG derivatives for protein PEGylation: PEG succinimidyl carbonate (a), PEG benzotriazole carbonate (b), PEG *p*-nitrophenyl carbonate (c), PEG trichlorophenyl carbonate (d), PEG carbonylimidazole(e) and PEG succinimidyl succinate (f).

The last compound of the 1st PEG generation is succinimidyl succinate (SS-PEG). It is prepared by reacting mPEG with succinic anhydride followed by the activation of the carboxylic acid to the succinimidyl ester. After conjugation with the protein the second ester linkage remains, which is very susceptible to hydrolysis. This in turn leads to the loss of the PEGylation benefits and to a residual succinate tag on the protein which may act as a hapten increasing its immunogenicity[112].

Many 1st generation PEGs activated as anhydrides, chlorides, chloroformates and carbonates, also react with hydroxyl groups. As already in high molecular weight PEGs the diol content can also be quite high reaching around 15% and therefore the use of these first generation PEGs is generally unsuitable for the conjugation to therapeutic proteins. The ability to generate an intermediate that can be purified from the unactivated PEG and to reduce the diol content generate more suitable approaches for protein modification[100] and was the driving force in the development of second generation PEG compounds.

2.2.4 The development of 2nd generation PEG Compounds

The development of 2nd generation PEG aimed at addressing the problems described for the 1st generation of these polymers. PEGylation, despite these flaws, still remains an important tool for improving drug delivery and is at the frontline of many drug therapies. This demand led the need for better PEG compounds to be developed and these compounds are classified as a 2nd generation of PEG compounds. They were designed to eliminate the high amount of diol found in high molecular weight PEGs, side-reactions and limited selectivity in substitution. One of the first PEGs of second generation is mPEG-propionaldehyde. It is easy to prepare in comparison to PEG-acetaldehyde, which is also is very susceptible to dimerization by aldol condensation. One of the key properties of mPEG-propionaldehyde is its selectivity under mild acidic conditions at ~ pH 5. The aldehyde is highly selective for the N-terminal α-amino group of a peptide chain, because of its lower pK_a compared to other nucleophiles.[113] The conjugation of the electophilic PEG to the amino groups in a protein is highly dependent on the nucleophilicity of each amino function. The nucleophilic attack will only take place when the pH of the solution is near or above the residues pK_a. Although complete selectivity is not observed, the extensive heterogeneity frequently seen with lysine is greatly reduced. The coupling of aldehydes to a primary amine happens by a condensation (Schiff base) reaction, which can be reduced to give a stable secondary amine linkage, Figure 2.9.

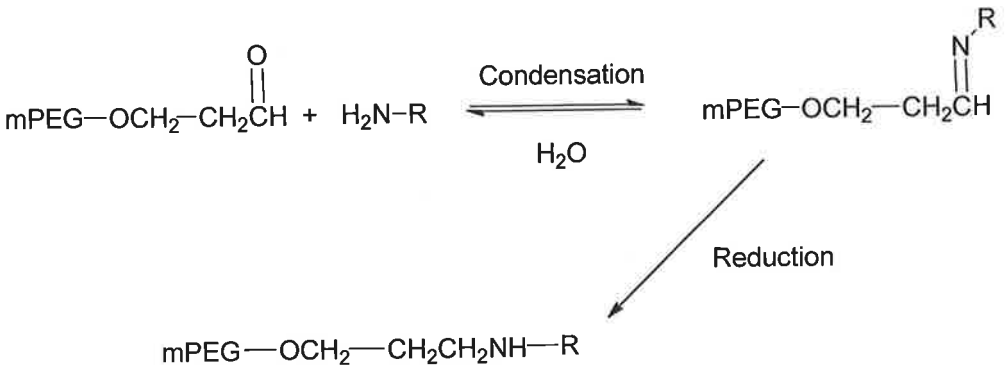


Figure 2.9: Reductive amination using PEG-propionaldehyde

An alternative to PEG-aldehyde is via the acetal derivative of PEG-propionaldehyde or PEG-acetalaldehyde. The aldehyde hydrate of acetal derivatives, and ultimately the free aldehyde, can be generated *in situ* by acid hydrolysis. Using the same method as for the free aldehyde derivates in Figure 2.9 above, the pH of the solution can be adjusted for protein modification. The main benefits of using the acetal derivative over the free priopionaldehyde or acetaldehyde, are the improved stability and higher purity of the modified PEGylated protein (Figure 2.10).

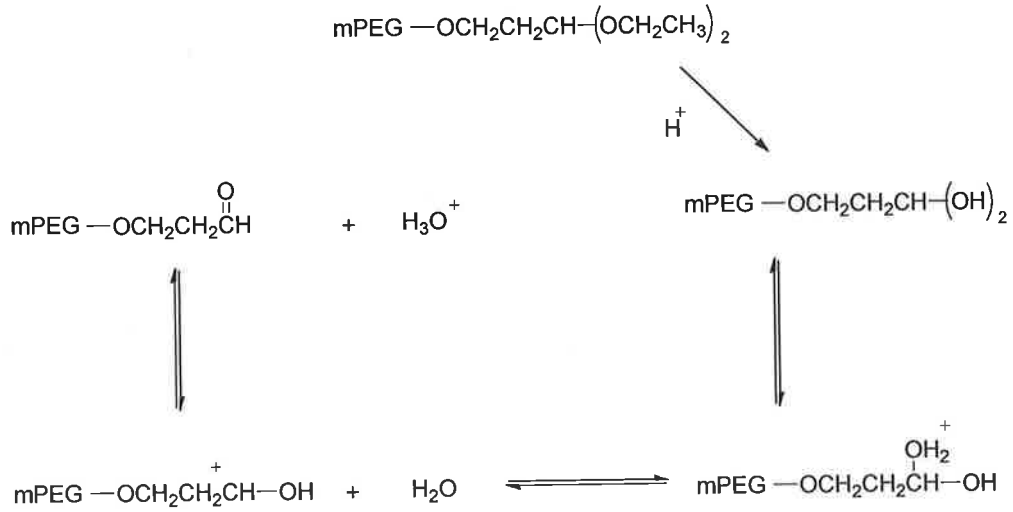


Figure 2.10: *In situ* generation of PEG-aldehyde for use in reductive amination

The most widely used group of acylating agents for protein modification are active PEG esters. The active ester can react with primary amines at a pH around

physiological conditions to form an amide bond. By generating the carboxylic acid intermediate, this allows the purification of PEG from monosubstituted and disubstituted impurities by ion exchange chromatography with compounds around 97% pure obtained in high yields. To transform the PEG-carboxylic acid to the succinimidyl active ester, the reaction is generally performed with *N*-hydroxysuccinimide (NHS or HOSu) and a carbodiimide (DIC or DCC).

Carboxymethylated PEG (CM-PEG) was the first carboxylic acid derivative to be introduced that did not contain a degradable linker to the PEG backbone, unlike other derivatives such as SS-PEG. The succinimidyl ester of the CM-PEG (SCM) is extremely sensitive to hydrolysis and therefore is difficult to handle. Harris and *et al.* decided to take full advantage of these more favourable kinetics and developed the propionic acid and butanoic acid derivatives of PEG[114]. Changing the distance between the active ester and the PEG backbone with the introduction of methylene units had a significant influence on amine reactivity in water, Figure 2.11.

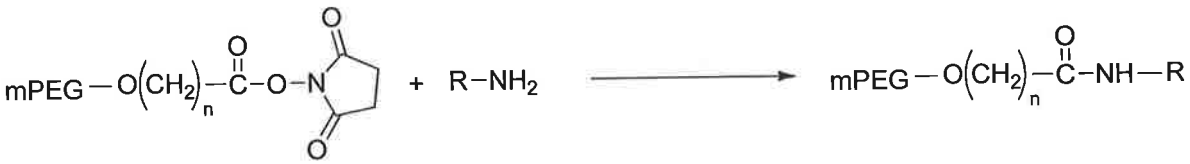


Figure 2.11: PEG NHS ester based on propionic acid (n =2)

2.2.4.1 Other PEG chemistries

There are many other types of PEG modifications such as thiol-reactive groups, oxidised carbohydrates or *N*-terminal serine or threonine, reversible and heterobifunctional chemistry. Thiol-reactive modification is site specific as it reacts with free sulphur groups on cysteine residues that are on the surface of proteins. Cysteine residues are a lot less common than lysine residues. However recombinant proteins can be produced with a cysteine added to their native sequences. As the cysteine content is low in proteins, site specific PEGylation can occur, minimising thereby the loss of biological activity and reducing immunogenicity. Problems encountered however with cysteine modification are the formation of disulfide bridges, incorrect folding of the peptide and also protein dimerization.

There are 4 main types of thiol reactive PEGs, developed for cysteine residues, each of them having their own advantages and disadvantages. PEG-maleimide (PEG-MAL),

Figure 2.12 (a) is highly reactive with thiols even under slightly acidic conditions (pH 6-7) but it is not stable in water as it can undergo nucleophilic attack. PEG-vinylsulfone (PEG-VS),

Figure 2.12 (b) reacts slowly with thiols and forms a stable thioether with the protein under slightly basic conditions, pH 7-8, but as the pH is increased, the faster the reaction will proceed. At elevated pH the PEG-VS is however susceptible to reaction with free lysine residues. PEG-iodoacetamide (PEG-IA),

Figure 2.12 (c), reacts very slowly by way of nucleophilic substitution which creates a stable thioether linkage. This reaction should be carried out with no exposure to light, to prevent the generation of free Iodine that may react with other amino acids. Orthopyridyl disulfide-PEG (PEG-OPSS),

Figure 2.12 (d), reacts specifically with sulfhydryl groups under both acidic and basic conditions (pH 3-10) to form a disulfide bond with a protein. Disulfide linkages are also stable, except in a reducing environment when the linkage is converted to thiols.

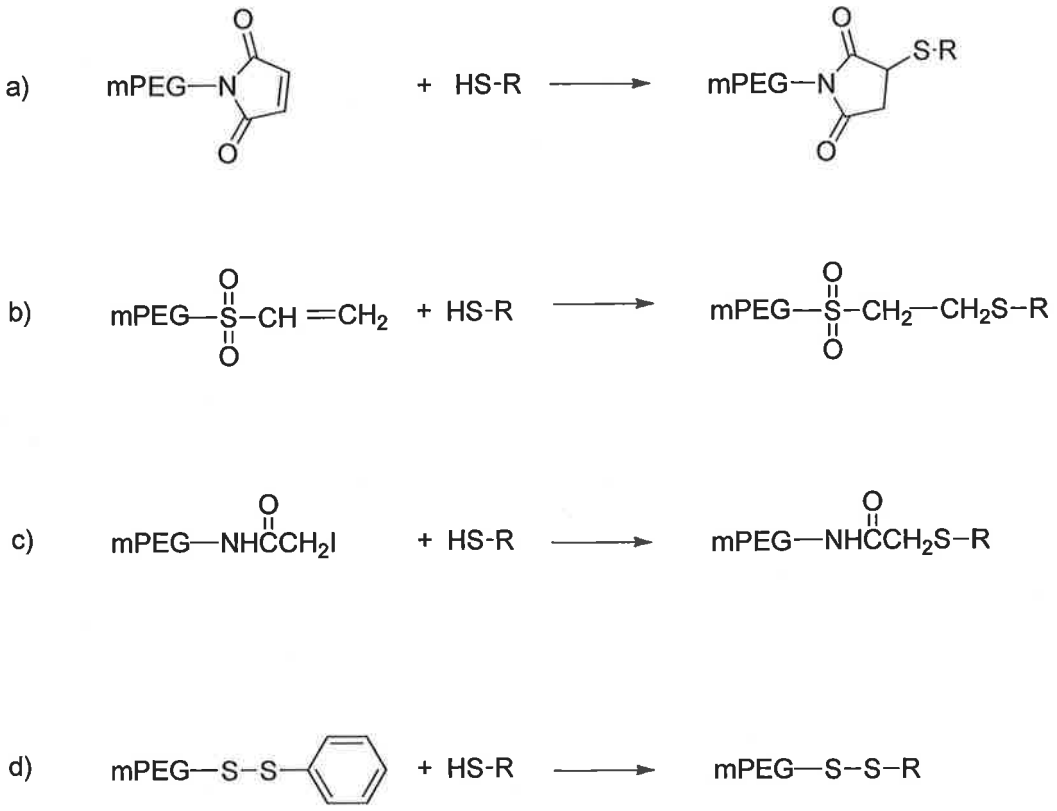


Figure 2.12: Thiol reactive PEGs. a) PEG-MAL b) PEG-VS c) PEG-IA d) PEG-OPSS

2.2.5 Site-specific PEGylation for oxidised carbohydrates and *N*-terminus

Site-specific PEGylation is also another method of PEGnology. Peg chemistry for oxidised glycoproteins or *N*-terminus serine or threonine is also another alternative

method of PEGylation for peptides and proteins. Different carbohydrates can be oxidised either by enzymes, such as glucose oxidase, or chemically with sodium periodate. The oxidation of carbohydrates generally creates multiple aldehyde groups which can be highly reactive. These can react easily with PEG-hydrazide to produce a hydrazone bond or with a PEG-amine through a reversible Schiff base reaction[115]. The hydrazone linkage may be reduced with sodium cyanoborohydride to a more stable alkyl hydrazide and the Schiff base may also be reduced to form a secondary amine. However, the amino groups present in proteins can also compete with the PEG-amine and this may cause the protein to aggregate by cross-linkage. The PEG-hydrazine affords better selectivity in this scenario. Under acidic conditions, around pH 5, all the lysyl amino groups will be protonated but not the PEG-hydrazide, because it is a weaker base ($pK_a \sim 3$, compared to approximately 10 for amino groups. Multiple attachment sites can be generated by this methodology.

Another option for site-specific PEGylation is to take advantage of a *N*-terminal serine or threonine which can be selectively converted by periodate oxidation to a glyoxylic derivative. Gaertner at al. successfully oxidized the *N*-terminal of a serine residue and then conjugated it to aminooxy or hydrazide PEG derivatives[116].

2.2.6 PEG chemistry for reversible PEGylation

Most PEGylation chemistry is designed to create a conjugate that contains a stable linkage to the protein. In most cases having a stable linkage to the protein is beneficial to long-term storage and facilitates purification. However, it is also generally observed that stable linkages to a protein can reduce their activity. This is possibly due to the steric crowding induced by the polymer at the active binding site of the protein. The molecular weight of the PEG is therefore an important factor in this regard. Higher molecular weight conjugates tend to have reduced activity *in vitro* but have better and improved pharmacokinetics *in vivo*. The opposite would apply to the activity and pharmacokinetic properties of lower molecular weight PEG conjugates. The overall aim of most conjugation techniques is to increase the circulation half-life without altering the activity. Reversible

PEGylation is a breakthrough technology developed to boost the half-life and biological activities of small molecule candidates as well as peptides and proteins without changing the mode of action of the therapeutic molecules and improving their pharmacokinetics. The worst problem in PEGylation is the fact that the polyethylene glycol polymers block the active sites of the therapeutic molecules. In reversible PEGylation, PEG polymers are attached to the drug with chain-like structures held in place by chemical bonds that gradually dissolve in the bloodstream, enabling the PEG molecules to detach in a measurable way from the drug. As they detach, the native molecules are released into the blood in a slow, constant, and predictable manner. An approach to regaining protein activity lost by PEGylation is the use of PEG chemistry that releases the native protein over time through enzymatic degradation, hydrolytic cleavage or reduction. The first such approach was SC-PEG which binds to histidine below pH 5, Figure 2.13.

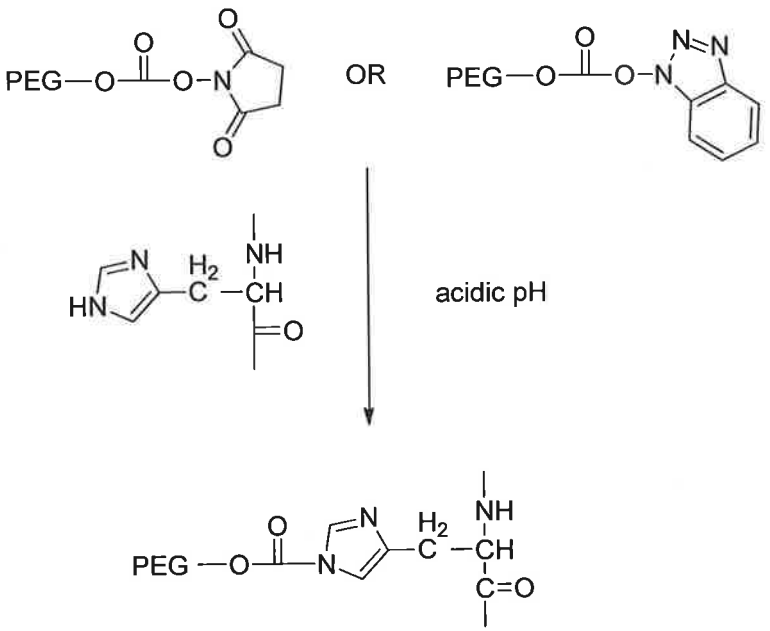


Figure 2.13: Attachment of PEG-benzotriazole of PEG-succinimidyl carbonate to histidine residues on proteins.

Double ester PEG reagents have also been investigated by Roberts *et al.* to control the release rates of the protein and regain activity over a period of time[105]. This was

achieved by esterification of PEG carboxyl (carboxymethyl, propionic, or butanoic) with hydroxyl acids to create a PEG acid that contains an internal ester linkage. (Figure 2.14)

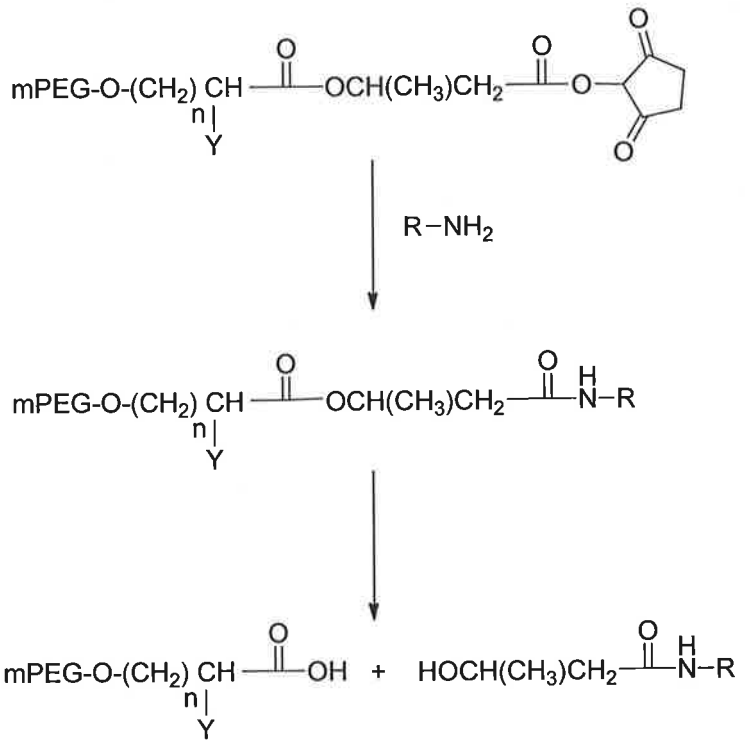


Figure 2.14: attachment and release of PEG-double ester from proteins (Y is an aliphatic or aromatic moiety)

The problem with the double ester PEG reagents is that they release the protein with a 'tag' that could lead to immunogenicity of the protein. Garman *et al.* used PEG maleic anhydride for PEGylation of proteins and the conjugates formed regenerated the native protein under physiological conditions and had also a 5-10 fold slower clearance rate than the native protein (Figure 2.15)[117]. Other examples of reversible PEG reagents synthesised by Bentley *et al.* were the mPEG phenyl ether succinimidyl carbonate and mPEG benzamide succinimidyl carbonate, used to conjugate to amino groups on lysozyme. Both reagents allowed reversible PEGylation and the rate of release of the free drug was controlled by the substitution position on the phenyl (Figure 2.16)[118].

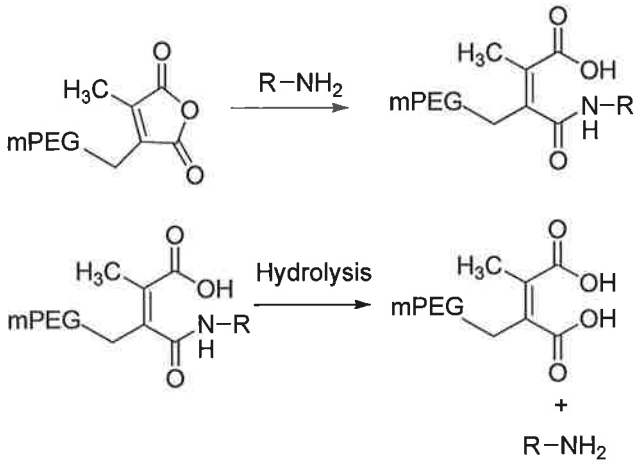


Figure 2.15: Attachment and release of PEG-methylmaleic anhydride from amine containing drugs

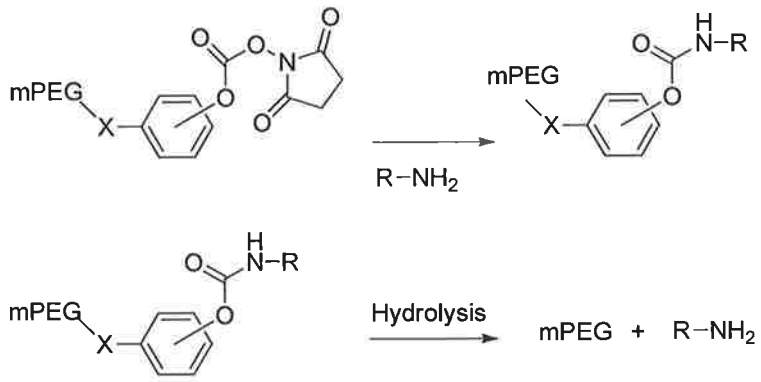


Figure 2.16: Attachment and release of PEG-phenyl NHS carbonates from amine containing drugs (X = O, NOCH-)

2.2.7 Heterobifunctional PEG chemistry

The development of a more and more sophisticated PEG chemistry has been accompanied by the generation of heterobifunctional PEGs. They are polymers which contain dissimilar terminal groups and have therefore the ability to attach via two different chemistries. This allows the conjugation of different macromolecules for various applications including immunoassays, biosensors, drug targeting, including liposome and virus modification, liquid phase peptide synthesis and many others. Several different heterobifunctional PEGs have been developed, with each of them having their own advantages and disadvantages. The end groups of the

heterobifunctional PEGs can be NHS esters, maleimide, vinyl sulfone, pyridyl disulfide, amine and carboxylic acids. However, the synthesis of heterofunctional PEGs is accompanied by the formation of the 2 symmetrical bi-functional PEGs.

A chromatographic approach has been used by Zalipsky *et al.* to purify a PEG acid that was synthesized by reacting glycine with SC-PEG derivative (Figure 2.8). Ion exchange chromatography was shown to be a very efficient purification method to separate monoacid from diacid and unsubstituted PEG. Bentley *et al.* prepared heterobifunctional PEGs by starting the polymerization reaction with benzyl alcohol, to produce benzyl PEG (Bz-PEG-OH). The hydroxyl group of the intermediate polymer is converted to a first reactive functional group[119]. The benzyl group can be removed by hydrogenolysis, without chemically affecting the first functional group. This allows the generation of a new terminal hydroxyl group for the addition of a second functional group. A promising strategy for the generation of heterobifunctional PEGs is the polymerization of ethylene oxide with an anion that ultimately becomes the end-group of PEG; this is currently the most direct route for heterobifunctional PEG synthesis. Terminating the reaction with water generates a hydroxyl group at the second terminal end that can undergo activation by chemical methods. For example, this method has been used by Yokoyama *et al.* to prepare a PEG with a hydroxyl at one terminus and an amino group at the other[120]. Cammas *et al* have also used this method to synthesise a PEG chain with an amino group at one terminus and a hydroxyl or methoxy group at the other terminus[121]. Nagasaki *et al* then synthesised a PEG compound with a formyl group at one terminus and a hydroxyl group at the other. However this method has its limits, because only those anions that are desirable as end groups are suitable for initiating polymerization for the synthesis of heterobifunctional PEGs by this route[122]. This method is also limited by the fact that no water can be present to avoid diol formation. This problem becomes more severe as the PEG molecular weight increases. If these problems can be resolved, generating heterobifunctional PEG reagents by anionic polymerization may remain the most efficient method.

2.2.8 Branched PEG structures

Branched structures have been found to be useful candidates for protein and peptide modifications. The first branched structure was developed by Inada *et al.* $mPEG_2$-chlorotriazine (2,4-bis(methoxypolyethylene glycol)-6-chloro-1,3,5-triazine (Figure 2.7). Following on from work carried out by Yamasaki *et al.*, producing branched PEGs based on a lysine core, Venerose *et al.* successfully constructed a highly purified branched PEG or "PEG2" using two linear PEG-BTC (or related PEG-SC) chains linked to the α- and ε-amino groups of lysine (Figure 2.17)[123].

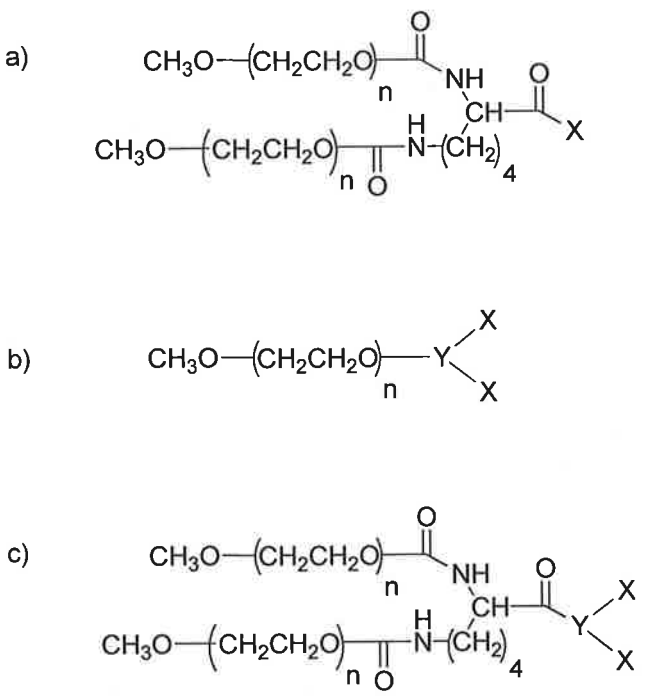


Figure 2.17: Branched PEG structures. a) branched PEG (PEG2), b) linear forked PEG, c) Branched forked PEG (X= molecule linker, functional group, Y= carbon branching moiety)

This allowed the modification of PEG chains upwards of 60 kDa and highly pure PEG to be synthesized with a single reactive group. PEG 2 (Figure 2.17(a)) is a very exciting tool for protein PEGylation. This is because PEG2 acts as a much larger entity than the linear PEG of same molecular weight. It also has the possibility of attaching two PEG chains at a single attachment site on the protein and reduce thereby the chance of protein inactivation. The PEG2 structure is also more effective at protecting proteins from proteolysis and at reducing their antigenicity

and immunogenicity. Another type of Branched PEG is the forked branched PEG (Figure 2.17 (b),(c)). Harris *et al* first synthesised a forked PEG by attaching to the terminus of a polymer backbone a single functional group linked to a trifunctional linker, such as a serinol or β-glutamic acid. The remaining proximal functional groups, which are attached to the central carbon atom, are able to react with two different molecules, which can be either the same or different, depending on their functionality. This can create a branched PEG chain that can also contain different molecules at a single terminus of its chain. Other PEG compounds have also been created as chelating agents for metal compounds. They can be used to precipitate proteins from solution and also to form ionic metal complexes. Forked PEG molecules are useful for conjugating molecules to have the two conjugates in close proximity to one another, *e.g.* for dimerization of cell surface receptors to activate cellular mechanisms. Another useful application for forked PEG molecules is to increase the loading capacity of small drug molecules.

2.2.9 PEG oligonucleotides

Oligonucleotides, in particular antisense oligonucleotides and aptamers, are now under active investigation as novel potential drugs because of their extremely high selectivity in target recognition. All of them however, share the problems of short half-life *in-vivo* because of either low stability towards exo- and endo- nucleases (present in plasma and inside the cells) and of their rapid excretion caused by their small size. Furthermore, their negative charge prevents their easy penetration into the cells which is required to exert their therapeutic effects. A PEG, bound to the hydroxyl group of a nucleic acid (directly[124] or through a spacer[125]), was found to increase its stability towards enzyme degradation, prolong its plasma permanence and enhance its penetration into the cells by masking the negative charges. A PEGylated aptamer, the 28-mer oligomeraptanib, has already been approved by the FDA for the treatment of age-related macular degeneration of retina. In this product, a branched PEG of 40kDa was attached to the oligonucleotide through a pentamino linker[126].

2.2.10 PEG as part of a drug delivery system.

PEG has been used for the delivery of many different organic molecules, proteins, peptides and anticancer agents. A few examples of PEG like polymers in delivery systems are styrene-maleic anhydride neocarzinostatin (SMANCS) copolymer, hydroxypropyl methacrylamide (HMPA) copolymer, dextran, polyglutamic acid and polyaspartic acid. These have all been used for conjugation to (bio)pharmaceuticals, to accomplish delivery in analogous ways. However, PEG conjugation has been most frequently approved for the clinical use in humans with therapeutic proteins than any other type of polymer. It offers a unique advantage of being a telechelic and semitelechelic polymer. (A telechelic polymer is a prepolymer capable of entering into further polymerization or other reactions through its reactive end-groups). There are many new strategies that have been developed for the delivery of several active classes of small molecules as PEG-Conjugates with the loading been monitored by various techniques such as UV, IR, NMR, Mass spectrometry and HPLC. Anticancer agents have benefited from this technology in particular and currently there are on-going investigations into other therapeutic fields.

The potential of proteins as medicinal agents have been highlighted for a number of years. The main problems with protein drugs are their poor circulating half-lives and low solubility. Therefore they have to be administrated at high doses to remain efficient. This in turn leads to the increased risk of triggering an adverse immune response. Advances in PEGylation to overcome these issues have been extended to the modification of smaller drugs.

2.2.11 PEGylation of low molecular weight therapeutic agents

There are many common problems encountered by anticancer agents of low molecular weight. They have low solubilities, are rapidly excreted from the body and have an untargeted biodistribution. PEGylation is a tool that can be used to overcome these problems. Generally, the properties of PEG are conveyed to the conjugated drugs and their body fate reflects that of the polymer. Increased

solubility, enhancement of pharmacokinetic properties and passive targeting have all been credited to PEG, and exploited with important drugs such as taxol, camptothecin, *cis*-platin and doxorubicin[127].

The conjugation chemistry for non-protein drugs faces fewer problems because of the reduced number of functional groups present on a small molecule, the absence of conformational constraints and the easier purification and characterisation steps for the drug polymer conjugates. For drugs of low molecular weight researchers are exploiting PEGylation for passive targeting to solid tumours, by the enhanced permeability and retention (EPR) effect[128], or simply for improving the drugs' pharmacokinetic profile by slowing down their clearance from the body. Usually the conjugates are designed as macromolecular prodrugs, because the drug has to be released to exploit its activity. In recent years, methods have been developed to release the drugs from the conjugate in specific cellular compartments and/or under controlled conditions. For example, intracellular drug release has been achieved by using special linkers or bonds between the polymers and the drugs. These might be either hydrolysed by the acidic pHs of endosomes (*i.e. N-cis* aconitic acid spacer and hydrazone linkages[129] or by lysosomal enzymes (*i.e.* H-Gly-Phe-Leu-Gly-OH or H-Gly-Leu-Phe-OH spacers[130]). A sophisticated strategy in the field of controlled-rate release of drug conjugates uses linkers that are designed to form double prodrug systems.[131,131a]

2.2.11.1 Linkers that respond to pH changes

These linkers are mainly used and designed for small drug conjugation. Following cell internalisation by endocytosis, the conjugates are exposed to the acidic pH of the endosome and lysosomes. Also it can be noted that the pH of intercellular tumour tissue is slightly more acidic than normal healthy tissues. These conditions can be exploited by using acid-labile spacers. The first acid labile spacer to be used was *N-cis*-aconitic acid which was used in conjugation with daunorubicin to aminoethyl polyacrylamide or poly-(D-lysine). In this case, the drug was released at a pH of 4 or lower, while in blood or at pH 6 the linker remained stable. A

hydrazone linkage can also take advantage of pH to release a biological cargo; this linker has been used in several different conjugates to release adriamycin or streptomycin from the carrier.

2.2.11.2 Linkers that are releasable by lysosomal enzymes

Linkers that are designed for release by lysosomal enzymes are mainly used in the delivery of small drugs. The releasable spacers are usually oligopeptides and they are promptly cleaved by lysosomal enzymes, thus enabling a lysosomotropic delivery of drugs. Lysosomal enzymes such as cathepsin B and D and other metalloproteinases play moreover a role in tumour growth and represent therefore attractive targets in drug delivery. Some examples of these linkers, which have been studied by Duncan *et al.* and Kopecek for optimization of drug release, include GFLG and GLGF, which appear to be the most effective[132].

2.2.11.3 Drug release by anchimeric-assisted hydrolysis.

This sophisticated strategy uses linkers designed to take part in a double prodrug release mechanism. The drug-linker is first released from the polymer by hydrolysis (first prodrug mechanism) and the linker triggers in turn the release of the free and active drug (second prodrug mechanism). Some examples of these double release mechanisms are the 1,6-elimination reaction or trimethyl lock lactonization (Figure 2.18).

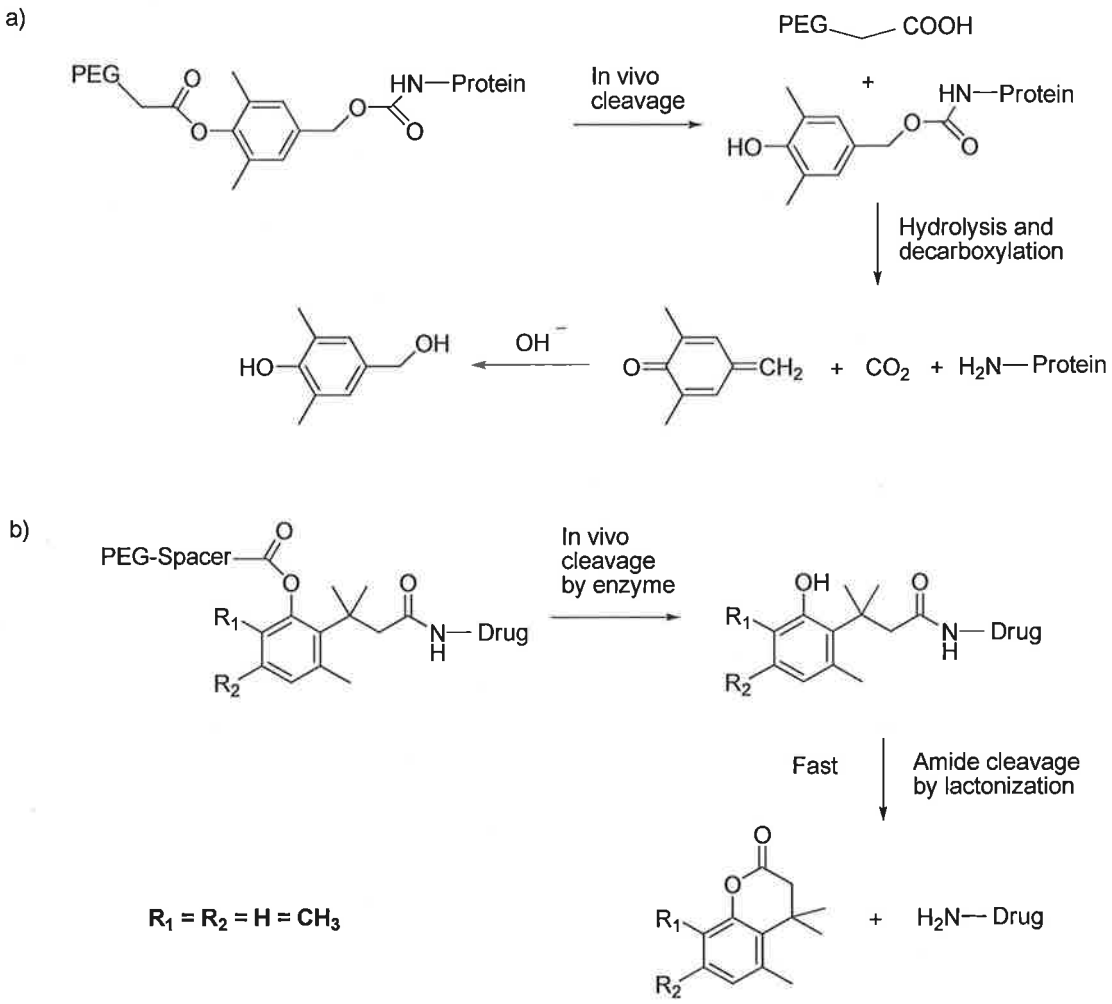


Figure 2.18: Drug control release from PEG chain; a) 1,6-elimination and b) trimethyl lock lactonization.

2.2.12 Chemotherapy and introduction to doxorubicin

The ability of a chemotherapy drug to kill cancer cells depends on its ability to halt cell division. Usually, the drugs work by damaging the RNA or DNA that assists the duplication of a cell during division. If the cells are unable to divide, they die by induction of the apoptotic pathway. The faster the cells are dividing, the more likely is a chemotherapeutic agent to kill the cells, causing ultimately the tumor to shrink. Chemotherapy drugs that affect cells only when they are dividing are called cell-cycle specific. Chemotherapy drugs that affect cells when they are at rest are called cell-cycle non-specific. The scheduling of the chemotherapy is set based on the type of cells, rate at which they divide and the time at which a given drug is likely to be

effective. This is why chemotherapy is typically given in cycles. Unfortunately, chemotherapies do not discriminate tumour from normal cells. Chemotherapy will kill all cells that are rapidly dividing. "Normal" cells will grow back but cumulatively side-effects occur. The "normal" cells most commonly affected by chemotherapy are the blood cells, the epithelial cells in the mouth, stomach and bowel, and the hair follicles, resulting in low blood counts, mouth sores, nausea, diarrhea, and/or hair loss. Different drugs may affect different parts of the body. Doxorubicin is a chemotherapeutic agent which is classified as an antitumor antibiotic. The latter are natural products produced by species of the soil bacteria *Streptomyces*. These anthracycline antineoplastic agents act during multiple phases of the cell cycle and are considered cell-cycle specific. However, there is a great need for targeted delivery of these chemotherapy agents to avoid damage of normal cells, eg. cardiotoxicity of doxorubicin. PEGylated liposomal Doxorubicin is a drug delivery technology recently introduced to address the toxicity problem associated with doxorubicin.

2.2.12.1 PEGylated Liposomal doxorubicin

PEGylated liposomal doxorubicin is a unique formulation of conventional doxorubicin in which a polyethylene glycol layer surrounds the doxorubicin–containing liposome. This property, combined with the longer half-life, promotes targeted drug delivery to the tumour site. During the drug design of these conjugates particular attention was given to parameters such as the molecular weight of the polymer (5-20kDa), its structure (linear or branched), and the choice of peptidic linker between the drug and its carrier (i.e., GFLG, GLFG, GLG, GGRR or RGLG) [133]. Surprisingly, the conjugate with the lowest molecular weight PEG displayed the highest antitumor activity (mPEG5000-GFLG-doxorubicin). This however is contradictory to the evidence that the candidate needs to be above molecular weights of 20-30kDa to allow accumulation of tumours by the EPR effect[134]. After further investigation into these results and analysis by light scattering showed that this low molecular weight PEG-Doxorubicin conjugates form micelles in solution which have an apparent molecular weight of 120kDa. This

supramolecular assembly ensures effective EPR effect and explains the higher antitumor activity of the conjugate. Many different Doxorubicin polymer conjugates have been designed with the linkers described above.

2.2.12.2 Mode of action of Polymer-drug conjugates

Increasing understanding of the mechanism of action of polymer-drug conjugates is guiding the design of a second generation of conjugates and the optimisation of their clinical applications. Hydrophilic polymer drug conjugates can be designed to delay their rate of clearance from the circulation *in vivo,* which is dependent on their molecular weight, and ultimately related to the rate of renal elimination,Figure 2.19 (a).

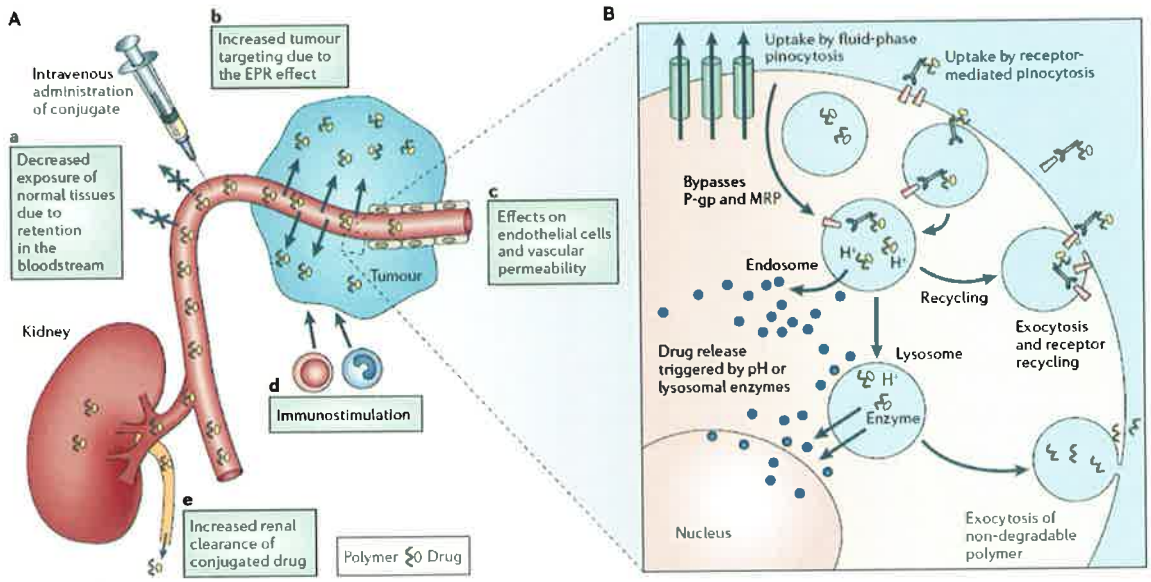


Figure 2.19: Mode of action of polymer-drug conjugates (figure from Nature Reviews, Cancer (2006) 6, 688)

After intravenous administration, Figure 2.19 (a) rapid blood clearance needs to be avoided to reach the high plasma concentration of the conjugate that is essential for the EPR effect to take place. If the polymer drug linkage is stable in the

bloodstream, the levels of free drug in plasma are low (>100-1,000 less compared to that of the free drug). Conjugates can circulate much longer than free drugs, which can lead to significantly increased drug accumulation in tumours. The blood concentration of the drug conjugate can indeed drive (passive) tumour targeting due to the increased permeability of the angiogenic tumour vasculature (Figure 2.19 (b)) (compared with normal vessels) which contributes to the EPR effect, (Figure 2.19 (c)). Through the incorporation of cell specific recognition ligands it is possible to exploit the added benefit of a receptor mediated targeting to tumour cells. It has also suggested that circulating low levels of conjugate, that are slow releasing, might lead to immunostimulation (Figure 2.19 (d)) [132].

When the polymer-drug reaches the tumour (Figure 2.19 (B)), it is internalized by tumour cells through fluid phase pinocytosis (in solution), receptor-mediated pinocytosis following non-specific membrane binding, due to hydrophobic or charge interactions, or ligand receptor docking. Depending on the linkers used, the drug will usually be released intracellularly on exposure to lysosomal enzymes or by a reduction of the pH; for example Gly-Phe-Leu-Gly and polyglutamic acid (PGA) are cleaved by the enzyme cathepsin B and a hydrazone linker degrades in endosomes and lysosomes in pH 6.5 - <4.5 ranges. By active or passive transport the released drugs can exit their vesicular compartments and ensure exposure to their pharmacological targets. Intracellular delivery can bypass mechanisms of resistance associated with membrane efflux pumps such as P-glycoprotein.

2.2.13 Immunogenicity and safety of PEGylated Proteins

PEGylation can normally reduce immunogenicity of proteins; there are examples of transforming immunogenic proteins into a tolerogen by PEGylation.[135] It is not easy generally to predict the characteristics of PEG-protein conjugates because they usually depend on the physicochemical properties of the protein, polymer and final conjugate. The likelihood of an immunogenic reaction can increase with the level of uric acid in patients leading to hyperuricemia. Unlike most mammals, humans lack an uricase enzyme, therefore, for therapeutic purposes, an enzyme totally foreign

to the human body (porcine origin) is used, and is PEGylated to prevent its immunogenicity[136]. However, during phase 1 clinical trials the formation of unusual anti-PEG antibodies was detected in some patients[137]. Presumably the methoxy group in the PEG chain at its terminus, remote from the linker to the protein[136], was identified as an antigenicity determinant which is rather surprising since methoxy end capped PEGs are generally used in modern marketed PEGylated biopharmaceuticals. No other reports on the mounting of anti-PEG immune responses following repeated administration of PEGylation liposomes[138,139] or PEG-glucuronidase can be found in the literature[140]. High levels of PEG used as an intravenous therapeutic agent *per se* have been shown to generate concentration and molecular mass dependent serum complement activation[141]; however, the quantities of PEG administered in PEGylated therapeutics are 10,000- to 1,000- fold lower. In toxicology studies, very high doses of PEG-Protein have been demonstrated to be capable of inducing renal and tubular vacuolization that is not associated with functional abnormalities and that disappears after the treatment[142]. Therefore, PEG-protein conjugates remain regarded as immunologically safe and non-toxic. It has also been demonstrated that potential protein immunogenicity can be better alleviated by attachment of larger and branched PEGs than by shorter and linear PEGs. In general, PEG and relatively low dosages of PEG-conjugates reduce the risk for an immunogenic response significantly[94]. A consequence of the conjugation however may be the formation of new epitopes, for example due to partial protein denaturation after conjugation or use of inappropriate spacers between the protein and the PEG chain. Hence, it is important to pay attention to suitable PEGylation chemistry, conjugation conditions and careful selection of the PEGylated site [100].

The size and shape of the PEG-protein conjugates determines their distribution and accumulation in the liver and other organs that are rich in reticuloendothelial cells. These include the spleen, lymph nodes, lungs and kidneys. The clearance of native or glycosylated proteins from these organs is lower for PEG-Protein conjugates than for their unconjugated counterparts. There are currently no reports of severe side-effects, but the consequences of life-long therapies with high doses of high

molecular weight conjugates are hardly predictable. Occasional warnings that significant PEG-protein accumulation in the liver may increase the risk of toxicity have appeared[94].

2.2.14 Limitations of PEG

PEG is made by chemical synthesis and like all synthetic polymers, it is polydispersed, which means that each batch is made up of molecules having incorporated different amounts of monomers, yielding to a Gaussian distribution of their molecular weights. This might lead to a number of drug conjugates that may have different biological properties. The polydispersity may alter the biodistribution of the conjugated molecule but also their immunogenicity. There have been many improvements in the development, and also in the purification methods, of PEGs in recent years with the polydispersity in PEG compounds reducing, but the problem must still be taken into consideration. This is particularly important when dealing with low molecular weight drugs, either peptide of non peptide drugs, where the mass of the linked PEG has more impact on the conjugates' characteristics[94]. Another problem associated with the use of polymers is their excretion from the body. PEGs are normally excreted in urine or faeces but high molecular weight compounds can accumulate, in particular in the liver leading to a macromolecule syndrome. It is not easy to extrapolate the kidney excretion limit by just looking at its usual threshold because other factors can play an important role. For example, PEG associates with a high number of water molecules which can increase its hydrodynamic volume up to 3-5 times that of a globular protein of the same molecular weight. This can affect the linear and flexible structure of PEG chains that help the polymer to cross the glomerular membrane and consequently the kidney excretion threshold. Urinary excretion is dependent on molecular mass and glomerular filtration has calculated cut-off at about 30kDa, higher molecular weights entities ending up in the faeces [143].

2.2.15 Conclusion

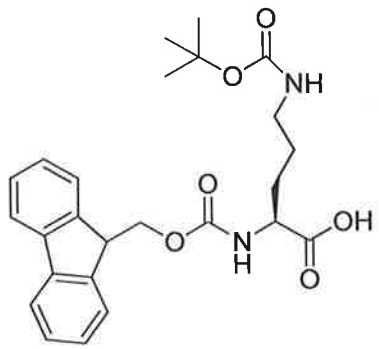
The array of PEG chemistries reviewed is among the dozens being used for clinical development of PEGylated peptides and proteins. The transition from first generation to second generation PEG chemistries is taking place at a rapid pace and future demand for PEG reagents will lead to new reagents for novel applications in the biopharmaceutical industry.

There are already hundreds of papers, patents and reviews on PEGylation of proteins, peptides and small drugs. This body of literature highlights the unique properties of PEG and shows that the technique has become extremely important even though PEG technology is still very young, with less than 30 years of existence. However from the large investigational work carried out to develop PEG reagents, there have only been a limited number of original advances or new chemical approaches. These studies are in fact directed to establish suitable applications of the numerous and therapeutically interesting proteins that otherwise exhibit unfavourable properties for clinical use. Currently, this is due to the increasing numbers of therapeutic proteins coming from proteomic discoveries.

2.3 Chemical synthesis of a dual-release polymeric prodrug.

2.3.1 Fmoc/*t*Bu peptide synthesis strategy

The peptide P18 was chosen as the HDP candidate for the synthesis of this multi-component drug delivery system as P18 is known to have anticancer activity and is selectivity towards cancer cells[85]. The peptide is used in many projects within the Devocelle group and has proven to be an excellent candidate and contains a broad spectrum of activity against many different cancer cell lines[144]. This hybrid peptide consisting of; cecropin A (1-8) and magainin 2 (1–12) is a cationic peptide made up of 18 amino acids. Its sequence can be assembled by Solid Phase Peptide Synthesis according to the Fmoc/*t*Bu strategy (Figure 2.22). Fmoc/*t*Bu is a protection scheme used in solid phase synthesis of peptide sequences, assembled from the C- to the N-terminus to minimise racemisation. This approach is also easily automated and has the ability to assemble large synthetic peptides quickly. The amino acids required to build the peptide by this strategy are purchased as Fmoc-protected amino acids (Example Fmoc-D-Lys-OH), Figure 2.20, that contain a base-sensitive protecting group on their N^{α}-amino group and an acid-sensitive side-chain protection based on a *t*-butyl group, for side-chain functionalised amino acids, along with a carboxylic acid group free to form an amide bond with the previous amino-deprotected residue.



Fmoc-D-Lys(Boc)-OH

Figure 2.20: Fmoc protected amino acid, Fmoc-D-Lys(Boc)-OH

During the assembly of the peptide by Fmoc/*t*Bu synthesis, the coupling reagents most commonly used are HBTU/ HOBt(1-hydroxy-benzotriazole)/DIEA or

HATU/DIPEA. HATU is regarded as being the most efficient as it speeds up the coupling and also reduces the loss of chirality integrity. However, until 2012 HATU was considerably more expensive as it was under licence making it not feasible to use for every synthesis.

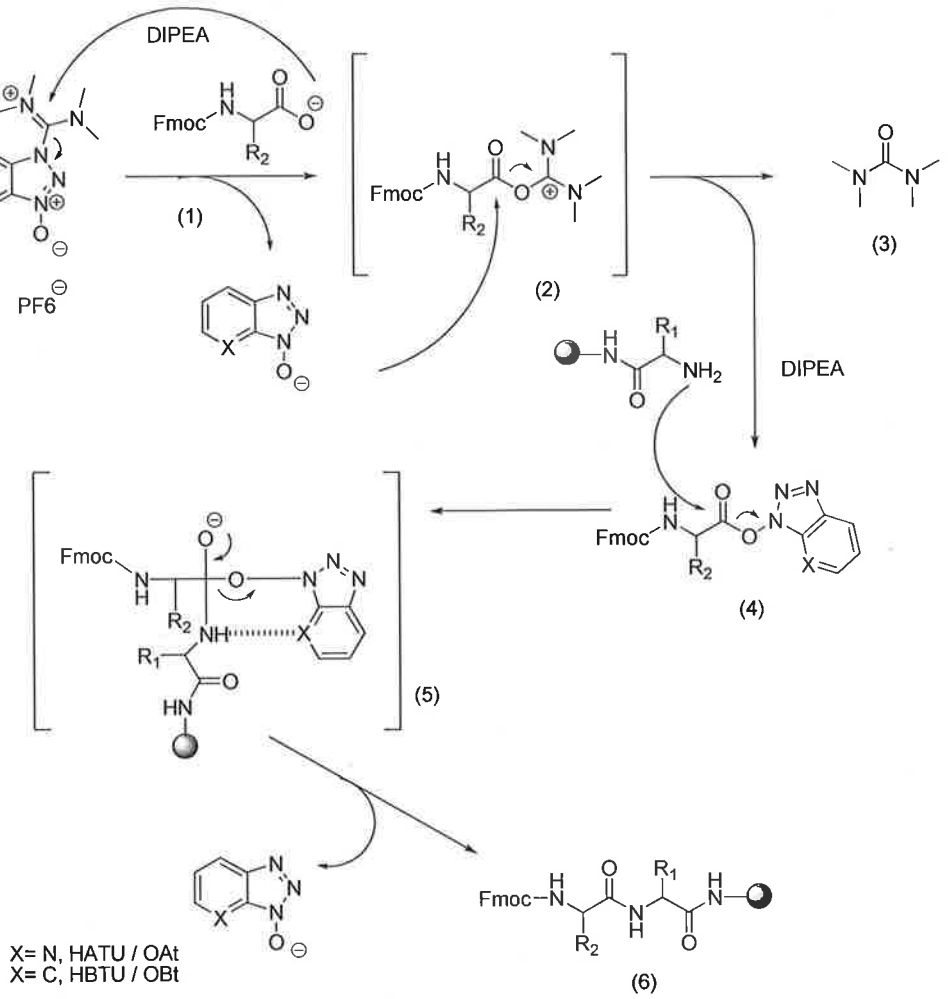


Figure 2.21: HATU/DIPEA coupling reaction mechanism

The mechanism of HATU/DIPEA or HBTU/HOBt/DIPEA mediated coupling occurs in two steps, activation and coupling. In the activation step, HATU or HBTU reacts with the carboxylic acid group, deprotonated by proton transfer to DIPEA, and forms an active ester intermediate and $^{-}$OAt as a side-product (1). HOBt is needed as an additive if HBTU is being used. $^{-}$OAt released reacts back in at the carbonyl group to form another active ester (2) with the elimination of the urethane side product (3). The second step in this reaction is the coupling of the active ester to form the

peptide amide bond on the solid support (4). This is where $^{-}$OAt is thought to have higher coupling efficiency than other coupling reagents as it is expected to create intramolecular hydrogen bonding by the neighbouring effect (5)[145]. This is caused by the nitrogen on the benzotriazole and the hydrogen of the reacting amide forming an N-H ionic hydrogen bond (5). This facilitates the coupling process by speeding up the amide bond formation making HATU one of the most effective coupling reagents for Fmoc/*t*Bu peptide synthesis. During the process of amide bond formation to form a resin-bound peptide, $^{-}$OAt is eliminated as a side-product (6). The general coupling and deprotection reactions implemented in the Fmoc/*t*Bu strategy can be seen in the peptide synthesis cycle below (Figure 2.22).

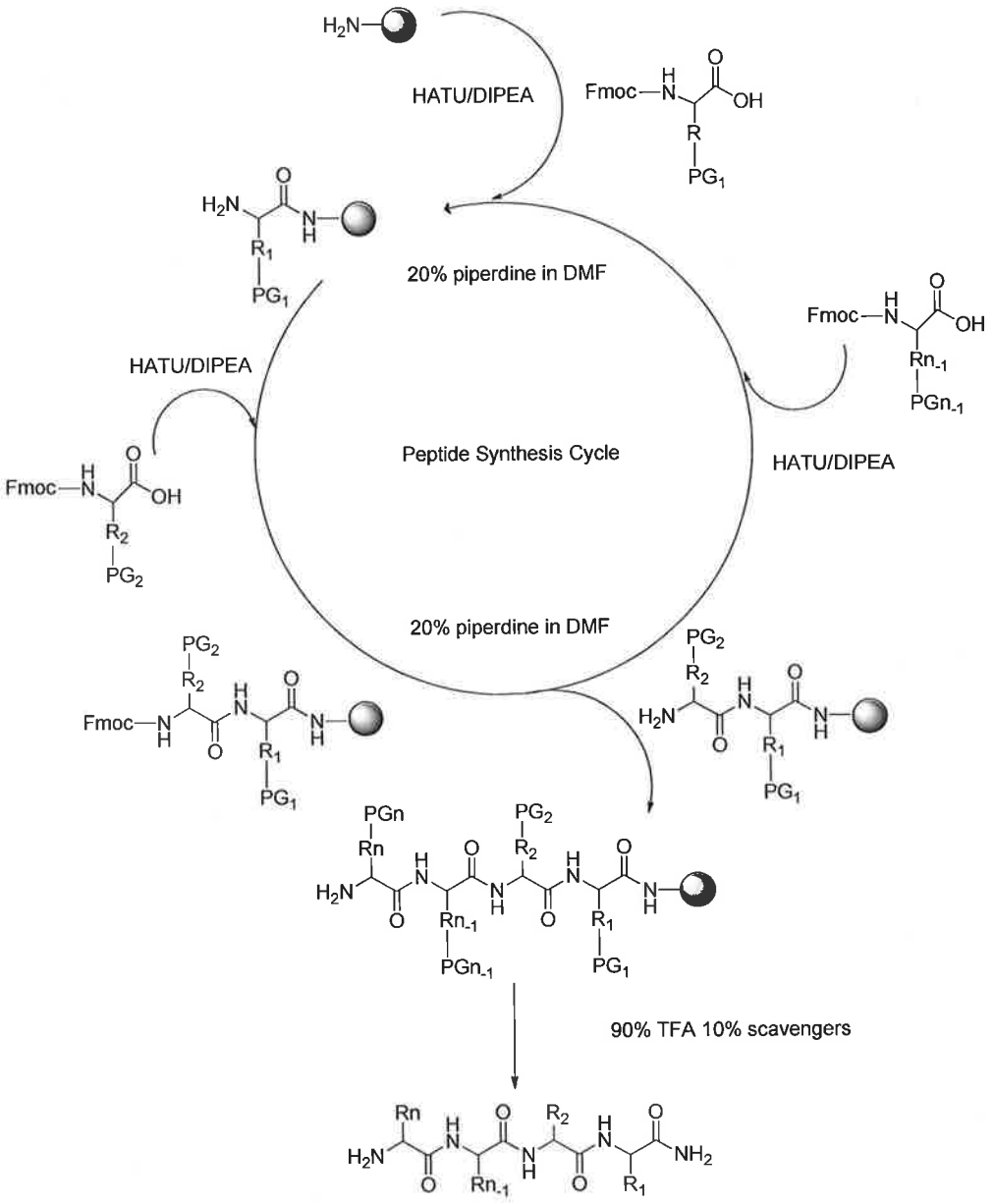


Figure 2.22: Fmoc/*t*Bu Peptide synthesis cycle

The Fmoc protected group is base sensitive and is typically removed by treatment with a solution of 20% Piperidine in DMF (Figure 2.23) product (A). It is very stable in acidic conditions but is swiftly removed under mildly basic conditions at room temperature. The mechanism for the reaction is an E1cb reaction, via the stabilized dibenzo cyclopentadienide anion; the dibenzo fulvene that is produced reacts in

turn with piperidine to give the product (B) leaving the peptide's amine group deprotected and free to react with the proceeding amino acid.

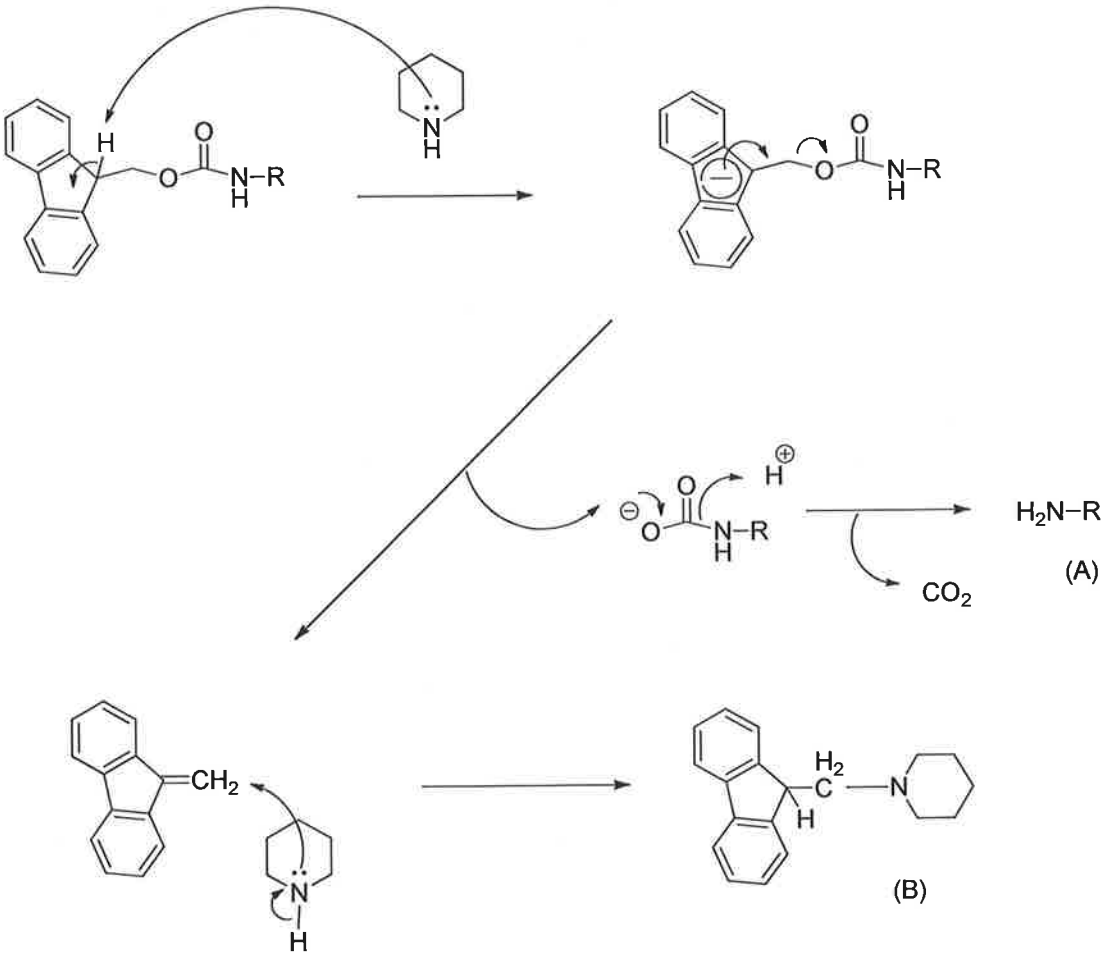


Figure 2.23: Fmoc deprotection mechanism

On the other hand, *t*Bu-based protecting groups, Figure 2.24 (Boc, *t*Bu - ether and – ester, trityl and Pbf) are used to protect amino side-chains in Fmoc/*t*Bu peptide synthesis and are removed by acidic treatment, Figure 2.25. The cationic species generated by these groups during deprotection require however the addition of nucleophilic scavengers to the acidic reagent.

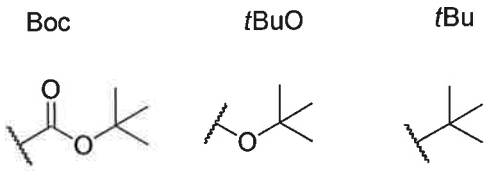


Figure 2.24: tBu-based protecting groups

The intermediate carbenium ions responsible for these side reactions are usually trapped by using scavengers. Depending on the amino acid and protecting group associated with that particular amino acid, different scavengers are used to stop *tert*-butylation on the amino acid side chains[146].

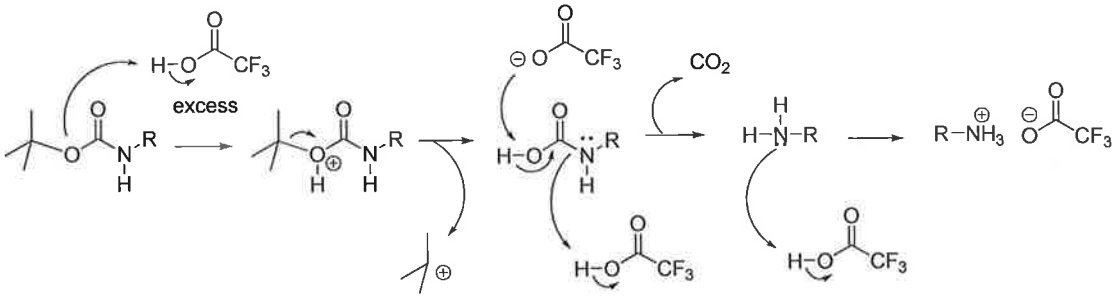


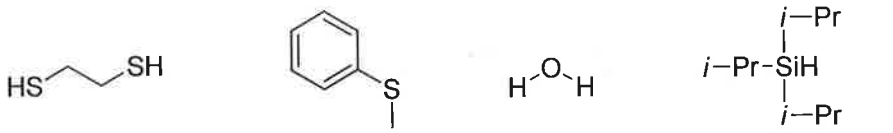
Figure 2.25: Mechanism of acidolytic cleavage of *tert*-butoxycarbonyl group

Shown below is a list of commonly protected amino acids for Fmoc/*t*-Bu synthesis and the scavengers whose presence is recommended during the removal of these protecting groups (Table 2.2).

Table 2.2: Amino Acid, side chain protection and cleavage scavenger

Amino Acid	Protecting Group	Scavenger
Ala	-	-
Arg	Pbf	H_2O/TIPS
Asn	Trt	H_2O/TIPS
Asp	*t*BuO	H_2O/TIPS/TA
Cys	Trt	H_2O/TIPS
Gln	Trt	H_2O/TIPS
Glu	*t*BuO	H_2O/TIPS/TA
Gly	-	-
His	Trt	H_2O/TIPS
Ile	-	-
Leu	-	-
Lys	Boc	H_2O/TIPS/TA
Met	-	EDT/TA
Phe	-	-
Pro	-	-
Ser	*t*Bu	TA/TIPS
Thr	*t*Bu	H_2O/TIPS/TA
Trp	Boc	EDT/TA
Tyr	*t*Bu	H_2O
Val	-	-

There are 4 main scavengers (Figure 2.26) used in the synthesis of a peptide such as P18 with the cleavage/deprotection time optimised at 2.5hours. Scavengers such as water, thioanisole (TA) and triisopropylsilane (TIPS) must be used in the isolation step from the resin in order to avoid side-reactions caused by intermediate carbenium ions in particular to prevent *tert*-butylation of the amino acid side-chains[146]. 1,2 Ethanedithiol (EDT) is also used in the cocktail cleavage for peptides containing Trp, Cys and Met. It prevents the *t*Bu group from attacking the sulphur group side-chains and also tryptophans' indole ring.[146] The cleavage cocktail for P18 consisted therefore of 90% TFA with 2.5% EDT, 2.5% TA, 2.5% TIPS and 2.5% H_2O. Under these conditions, there was no evidence of a *tert*-butylation of the peptide as verified by mass spectrometry.



1,2 ethane dithiol	thioanisole	water	triisopropylsilane
EDT	TA	H_2O	TIPS

Figure 2.26: Common scavenger reagents

With the optimal composition of the cleavage cocktail predicted for the isolation of the P18 peptide on the basis of its sequence, the first step in the synthesis pathway of the peptide was then to determine which resin to use on the solid support.

2.3.2 Sieber amide resin approach

Sieber amide resin is a part of a group of amide resins which offers an amide on the C-terminus of the peptide once cleaved from the resin support. Sieber resin contains a PEG spacer attached to the polystyrene bead which allows elongation of medium sized peptides > 20amino acids and prevents the formation of truncated peptides and the folding of the peptide chain on itself[147]. It was selected originally to facilitate the selective off resin PEGylation of a peptide with a free N^{α}-amino terminus, but otherwise fully protected. The Fmoc/*t*Bu elongation procedure of P18 from the Sieber resin can be seen in (Figure 2.27) and how this solid support has an acid labile bond that can be cleaved from the resin under mild acidic conditions which leave the side-chains of the amino acids protected. This allows for the selective coupling of the PEG on the N-terminus in solution and overcome the poor diffusion of large polymer chains in the resin when the PEGylation is performed on a supported peptide. However crude yields of P18 isolated after cleavage of the peptide from the Sieber resin were too low to consider this to be a viable route for the complete synthesis. At this stage, it was decided to synthesise a short model peptide to investigate the synthetic pathway of PEG attachment.

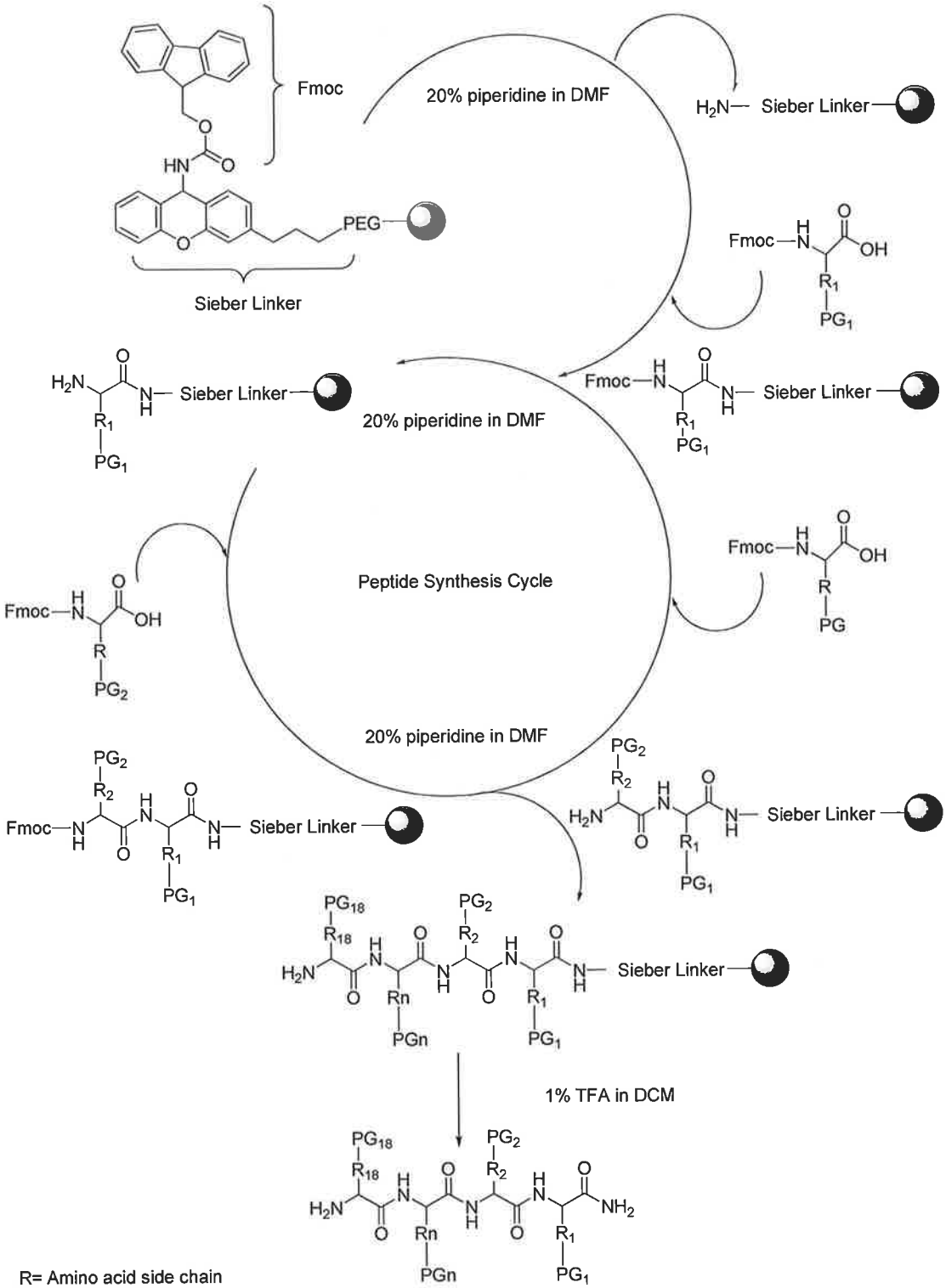


Figure 2.27: Fmoc/*t*Bu coupling strategy of P18 on Sieber resin

2.3.3 PEGylation on solid support

An approach based on a solid phase route, including the attachment of the PEG directly onto the solid support via the free N-terminal amino group, was investigated as an alternative method to produce the PEGylated peptide. At this development stage, it was decided to firstly work on the methodology for the complete synthetic route of the multi-component drug delivery system, combining the peptide, a classical anticancer agent and a polymer. A short peptide sequence and also a PEG of reduced molar mass were initially selected to optimize the reactions and demonstrate the feasibility of the approach.

2.3.3.1 Synthesis of peptide GRGDS

Peptide GRGDS (compound 5.2.1.2) is a short peptide used to target anticancer agents to the tumour vasculature, with the RGD (integrin binding) motif inbeded in the middle of the sequence. The peptide was synthesised by automated synthesis according to the Fmoc/*t*Bu strategy. The solid support used for this peptide was a Rink amide MBHA resin. The linker of this resin is acid sensitive and cleaved by high concentrations of TFA, up to 95%. Once the peptide is cleaved from the resin, the C-terminus of the peptide is left as an amide. This generates a peptide with a non-natural C-terminus, therefore resistant to exopeptidases hydrolysing C-terminal residues. However, unlike the Sieber resin, the peptide is fully deprotected by the higher percentage of TFA. Therefore the PEG must be added on the resin to avoid any coupling of the PEG to amino acid side-chains. The GRDGS peptide was synthesised and an aliquot of the resin was cleaved to confirm the successful assembly of this sequence. The cleavage cocktail contained 90% TFA and TIPS, TA and H_2O where used as the scavengers due to the presence of arginine, aspartic acid and serine in the peptide sequence. The correct peptide was identified by electron spray mass spectrometry.

2.3.3.2 Attachment of Fmoc-NH-PEG_{20atom}-COOH to peptide H-GRGDS -

The short bifunctional PEG linker Fmoc-NH-PEG_{20atom}-COOH was used to modify the N-terminus of the resin bound GRGDS peptide. The short peptide was successfully amidated using HBTU/ HOBt/DIEA coupling chemistry and the reaction completion was monitored by the qualitative Kaiser test. The latter indicates the presence of a free N^{α}-amino group (positive test) following Fmoc deprotection or its absence (negative test) *e.g.* the successful addition of a Fmoc-protected amino acid or the unsuccessful deprotection of the N-terminal protecting group.

2.3.3.2.1 Kaiser Test

The Kaiser test reagents include ninhydrin and phenol, both in ethanol and potassium cyanide in pyridine. One drop of each reagent is added into a test tube containing a small amount of the resin beads and heated to 110 °C for 3 minutes. In a negative test, the ninhydrin does not react and the resin beads remain colourless. Conversely, in a positive test, the ninhydrin will react with the free amine and turn the beads a purple colour. The ninhydrin undergoes hydrolysis and reacts with the free amine group on the peptide. The peptide in turn undergoes a rearrangement and with the loss of the amine group. The dark purple colour indicates the presence of a free amine group whereas a clear/yellow colour will indicate that there is no free amine present, Figure 2.28.

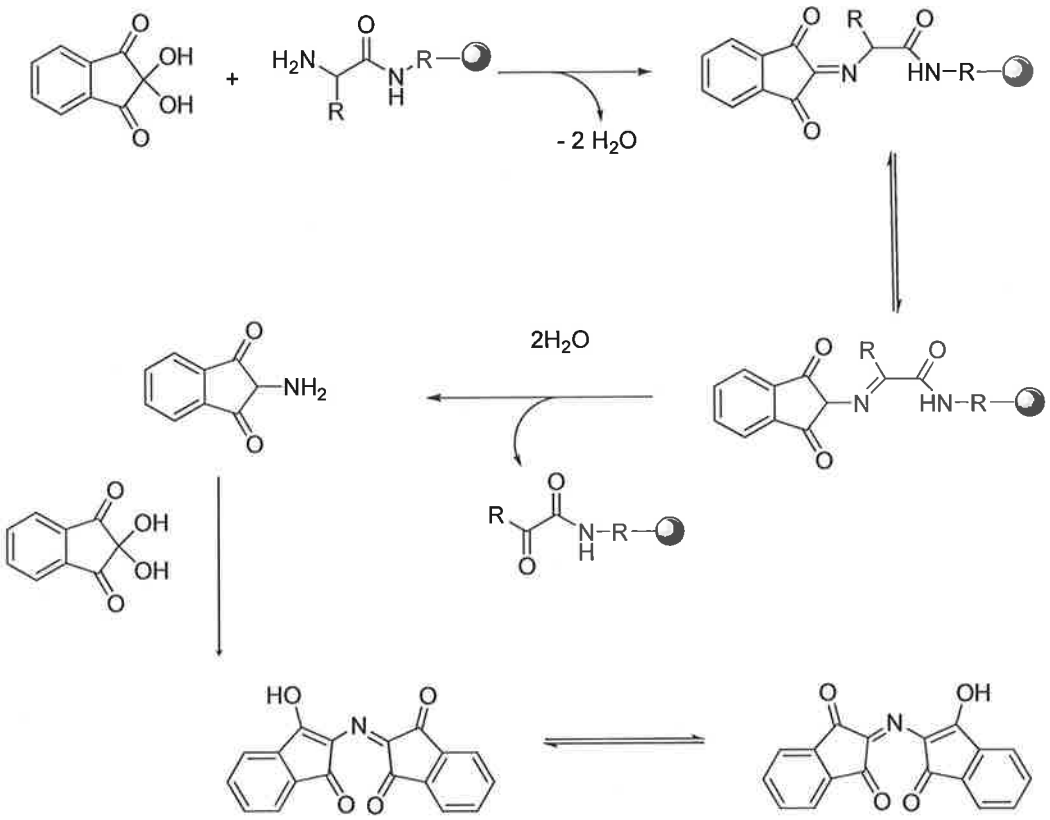


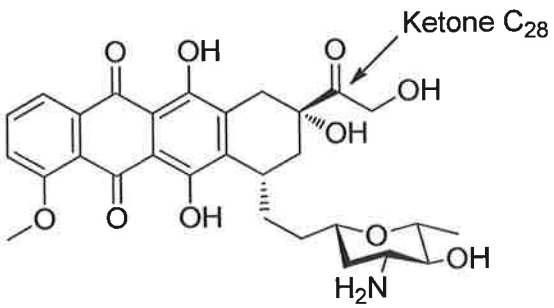
Figure 2.28: Mechanism of Kaiser Test

During the synthesis, the Fmoc protecting group was removed by 20% piperidine in DMF. This was also monitored by the Kaiser test to ensure complete deprotection. A short PEG was used to determine the synthetic pathway but it was anticipated that the addition of larger PEG molecules would require a different support, as the pore size of a MBHA Rink amide resin would not allow the diffusion of large PEGs in this support. An Amide PEGA resin will be used as a substitute in this case.

2.3.3.3 Introduction of *S*-benzyl thiosuccinic acid

In order to combine the PEGylated peptide to the DOX via a hydrazone bond, to allow a pH triggered release mechanism, a short organic linker had to be synthesized to promote the selective hydrazone formation between a hydrazide-functionalised peptide and the ketone of DOX. The short hydrazide linker is designed to join the PEGylated peptide to the DOX but also to enable the release of

DOX under acidic conditions due to the changes of pH within the lysosome of the cell, pH 7.6 to pH 4.5[95]. The DOX is conjugated via the ketone at the C28 position (Figure 2.29) by a hydrazone bond.



Doxorubicin

Figure 2.29: DOX

Although this is not the only carbonyl group available in the molecule it is the most reactive one due to the other groups being stabilised and conjugated in a ring. The hydrazide group was formed on the supported peptide by its reaction with *S*-benzyl thiosuccinic acid (Compound 5.2.1.1) and subsequent replacement of the *S*-benzyl group as a hydrazide (compound 5.2.1.5).

2.3.3.4 Synthesis of *S*-benzyl thiosuccinic acid

The starting material for the *S*-benzyl thiosuccinic acid was succinic anhydride. Succinic anhydride was reacted with benzyl mercaptan in the presence of DMAP as a catalyst. (Figure 2.30)

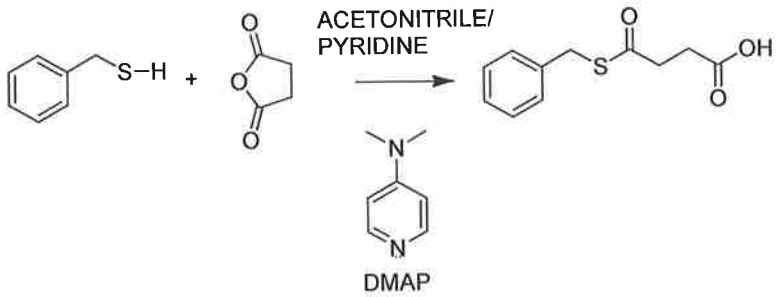


Figure 2.30: Synthesis of *S*-Benzyl thiosuccinic acid

DMAP is a strong base and reacts more rapidly than other bases, such as pyridine (also present), due to the resonance stabilization effect by the *p*-dialkylamino group. The DMAP reacts with the succinic anhydride to facilitate the ring opening (Figure 2.31).

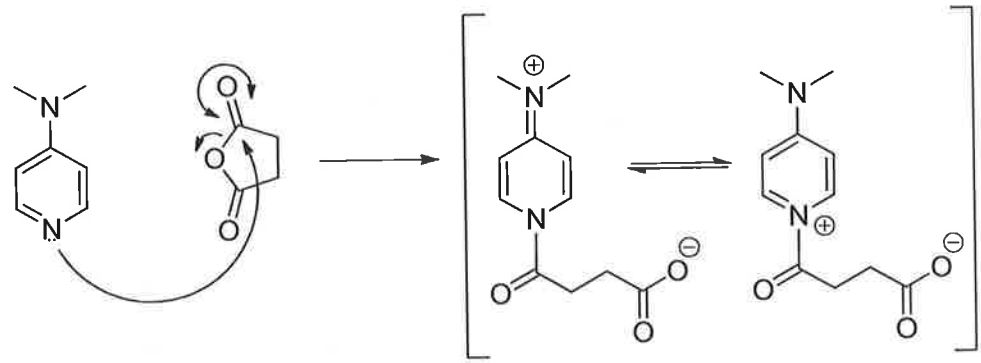
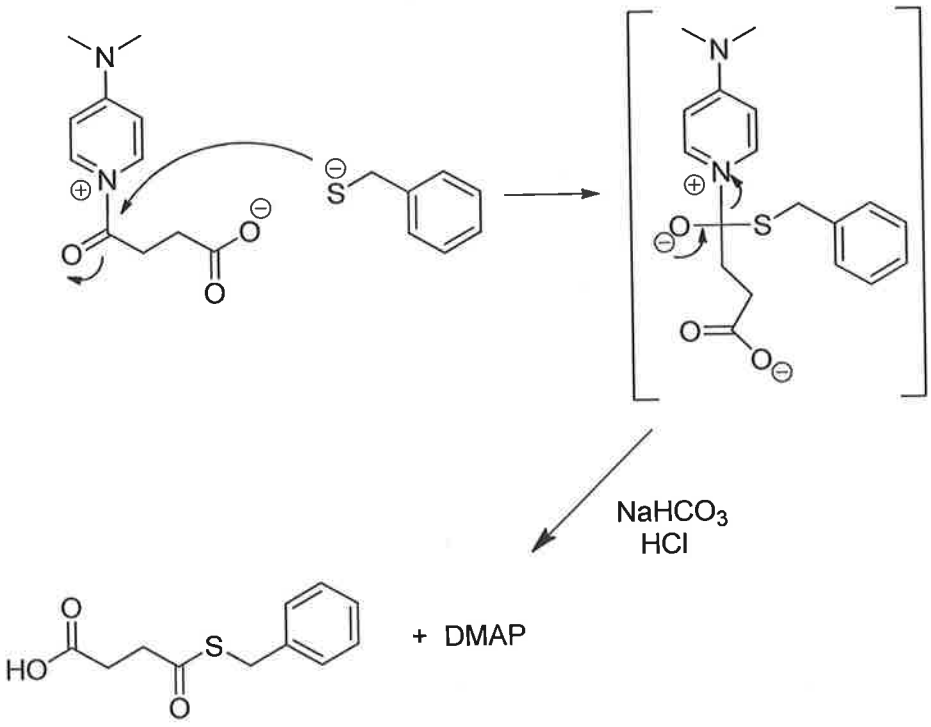


Figure 2.31: Ring opening formation of succinic anhydride

This allows the sulphur group on the benzyl mercaptan to act as a nucleophile and form a tetrahedral intermediate with the activated carbonyl group of the acylated DMAP[148].



2.32: Formation of S-benzyl thiosuccinic acid

The final product was then extracted with 5N HCl and isolated as a white precipitate without any need for further purification (Figure 2.32). The compound 5.2.1.1 was confirmed by ^{1}H NMR. (Appendix 1)

2.3.3.5 Coupling of *S*-benzyl thiosuccinic acid

The *S*-benzyl thiosuccinic acid was added by using HBTU/HOBt with DIEA as coupling reagents in 5 molar excess to the supported, PEGylated, GRGDS peptide (compound 5.2.1.3), forming an amide bond between the PEG amino and the *S*-benzyl thiosuccinic acid's carboxyl group. The reaction was monitored by the Kaiser test to ensure complete coupling.

2.3.3.6 Formation of the hydrazone bond

Hydrazine hydrate, as a 1:1 solution with 1,4 dioxane, was added to the resin-bound PEGylated GRGDS peptide, N-terminally modified with a thioester, in a reaction vessel and placed on a shaker for 4 hours. The lone pair on the nitrogen on the hydrazine hydrate attacks the carbonyl group of the benzyl thioester according to a S_N2 reaction. The reaction favours this site over the amide's carbonyl site due the stability of the amide bond, the thiol generated from the thioester being a better leaving group (compound 5.2.1.5) (Figure 2.33).

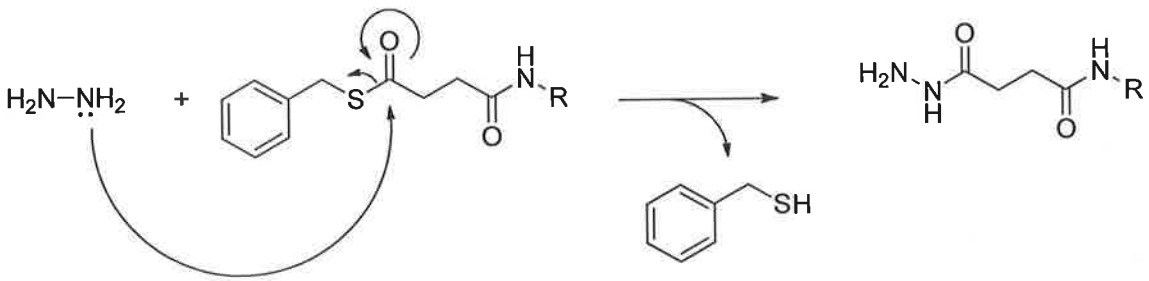
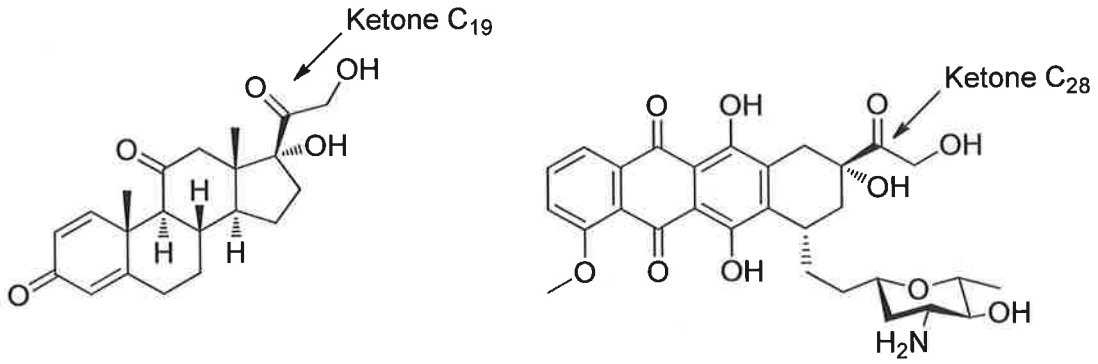


Figure 2.33: Hydrazide formation

Once formed the hydrazide group can be used in a 'click' chemistry type of reaction to selectively bind to a ketone via a hydrazone bond. The reaction with the hydrazide was repeated twice to ensure complete coupling. After this stage of the synthesis the peptide can be cleaved from the resin and chromatographic purification and mass spectra analysis carried out before the addition of the anticancer agent.

2.3.3.7 Ketone hydrazone formation

Due to the high cost of doxorubicin, prednisone was used initially to investigate the formation of the hydrazone bond. Prednisone is a steroid drug which contains the same functional ketone reactive site as doxorubicin and is an ideal candidate as a replacement for the feasibility study (Figure 2.34).



Prednisone Doxorubicin

Figure 2.34: Structure of Prednisone and Doxorubicin with Ketone identification

Prednisone was added to the hydrazide-modified peptide in anhydrous methanol and left to stir for 24hours under an argon atmosphere; the conjugation of Prednisone was confirmed by MALDI TOF (Table 2.3).

Table 2.3: Mass Spectrometry confirmation of PEGylated peptide conjugate with Prednisone

Peptide	Expected Mass	Mass Found
H-GRGDS-NH_2	489.4801	490.3
Bn-*S*-$C_4O_2H_4$-GRGDS-NH_2	1014.4077	1014.6
N_2H_3-$C_4O_2H_4$-GRGDS-NH_2	921.4152	922.6
Prednisone-N_2H-$C_5O_2H_4$-GRGDS-NH_2	1261.8432	1262.4906

With the synthetic pathway completed and the final product confirmed by Mass Spectrometry, the peptide P18 and larger PEG moieties can be substituted into the pathway for the GRGDS and 20 atom PEG respectively.

2.3.3.8 P18 peptide synthesis on Rink Amide PEGA resin

Amide PEGA resin was chosen as the solid support for larger PEG molecules. The PEGA resin developed by Merck Novabiochem can support the use of large macromolecules, up to 30k Da, due its PEG backbone attached to the polystyrene beads. The PEGA resin also has large swelling properties and is available pre-swollen in methanol to ensure maximum loading onto the resin. This allows for the PEGylation of resin-bound peptides, synthesised furthermore with minimal folding or truncating of the peptide itself. It also has a low substitution to allow maximum attachment of these larger molecules. The peptide P18 was synthesised by automated Solid Phase Peptide Synthesis using HOBt/HBTU or HATU coupling reagents, with DIEA as a base. During the P18 chain elongation, double coupling procedures (double the equivalents of amino acid and coupling reagents by repeating the coupling step) had to be applied for Leu^8 after the Pro^9 amino acid in the sequence (Figure 2.35). This is due to the steric nature of the proline amino acid and difficulty in coupling to a secondary amine as the N-terminus.

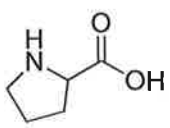
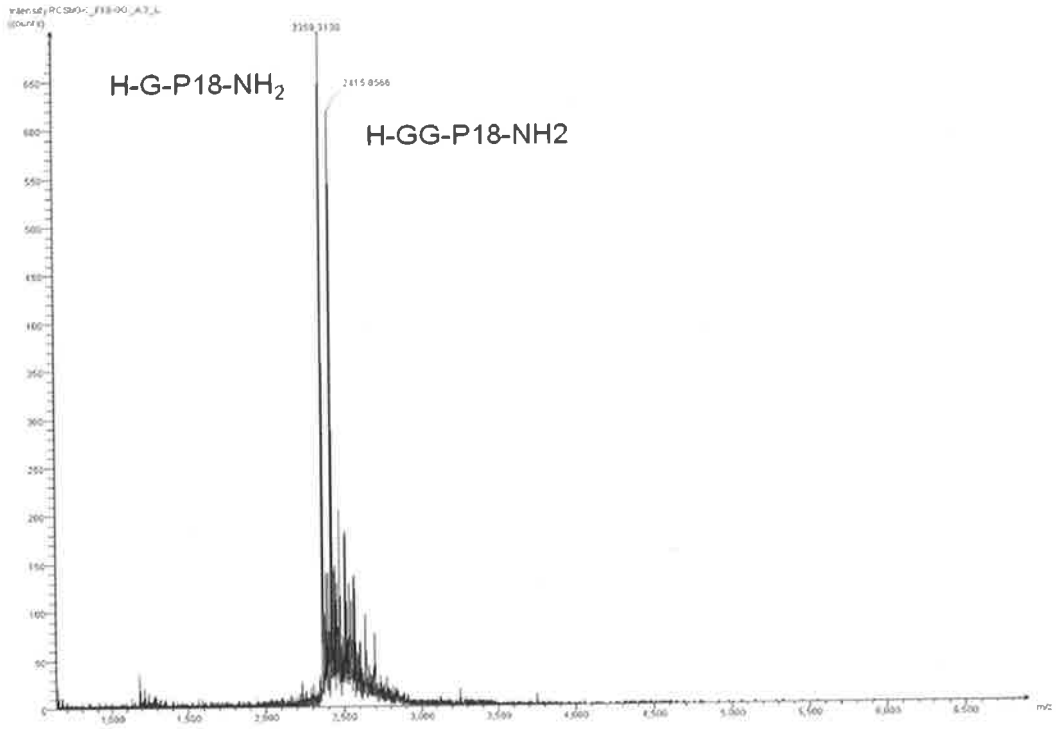


Figure 2.35: Proline amino acid residue containing a secondary amine

The coupling was followed by N^{α}-Fmoc deprotection. The level of deprotection can indirectly indicate how successful the previous coupling reaction was, by UV-monitoring of the dibenzofulvene formed during deprotection. If the sequence after Pro^{9} isn't elongated by a double coupling protocol (20 equivalents of amino acids overall), the UV monitoring will show this difference and incomplete coupling of Leu^{8} will arise with formation of a deletion peptide. Proline is indeed unique as it is the only one of the natural 20 amino acids that contains a secondary amine and gives inconclusive results by the Kaiser test. The automated synthesizer can follow by UV-detection the Fmoc deprotection reaction at every cycle and provide a direct monitoring of this reaction and indirect monitoring of the coupling reaction.

2.3.3.9 Addition of GFLG protease-sensitive linker

The Gly-Phe-Leu-Gly peptide linker which represents a substrate of the Cathepsin-B protease was added at the N-terminus of the peptide P18 (compound 5.2.1.8), by automated synthesis. Each amino acid of this linker required a double coupling procedure. This is due to the increasing hydrophobicity of the peptide as the sequence is elongated and the concomitant occurrence of interchain association effects. When a single coupling procedure with 10 equivalents of amino acids is used, deleted peptides are formed, as evidenced by mass spectrometry. Even the addition of a simple glycil-glycil linker, for the synthesis of a negative control which cannot be processed by Cathepsin-B failed when a single coupling chemistry was implemented. The MALDI-TOF mass spectrum clearly shows the formation of a deletion peptide.(Figure:2.36)



2.36: MALDI TOF spectrum of deletion peptide H-G-P18-NH_2

2.3.3.10 Addition of Fmoc-NH-PEG_{2000}-COOH to resin-bound GFLG-P18

The free N-terminus of the resin-bound P18 peptide elongated with the GFLG linker, compound (5.2.1.8) was amidated with Fmoc-NH-PEG_{2000}-COOH. The latter reaction was performed by using HATU/DIEA coupling chemistry. HATU reacts much quicker than HBTU/HOBt and was found to be a more efficient coupling reagent for the addition of the PEG, requiring a lower number of equivalents of PEG during the coupling reaction. This shows a reduction from 5 equivalents using HBTU/HOBt to 2 equivalents using HATU.

2.3.3.11 Addition of *S*-benzyl thiosuccinic acid and hydrazide bond formation.

The addition of the *S*-benzyl thiosuccinic acid and formation of the hydrazide bond proceeded without difficulty and each step was monitored by small scale cleavage and mass spectrometry (Table 2.4). The matrix used to determine the mass of the compounds by MALDI TOF was α-cyano 4-hydroxycinnamic acid at a ratio of 5:1 matrix to sample for PEGylated compounds and 1:1 for the non-PEGylated compounds.

Table 2.4: Mass Spectrometry analysis of the peptide components of the multicomponent drug delivery system

Peptide	Expected Mass	Mass Found
H-P18-NH_2	2300.4760	2299.8745
H-GFLG-P18-NH_2	2674.6719	2673.3981
Fmoc-NH-PEG_{2000}-GFLG-P18-NH_2	5023	5024.0430
NH-PEG_{2000}-GFLG-P18-NH_2	4800	4801.2998
Bn-S-$C_4O_2H_4$-NH-PEG_{2000}-GFLG-P18-NH_2	5053	5052.4160
N_2H_3-$C_4O_2H_4$-NH-PEG_{2000}-GFLG-P18-NH_2	4933	4933.0356

2.3.3.12 Addition of doxorubicin

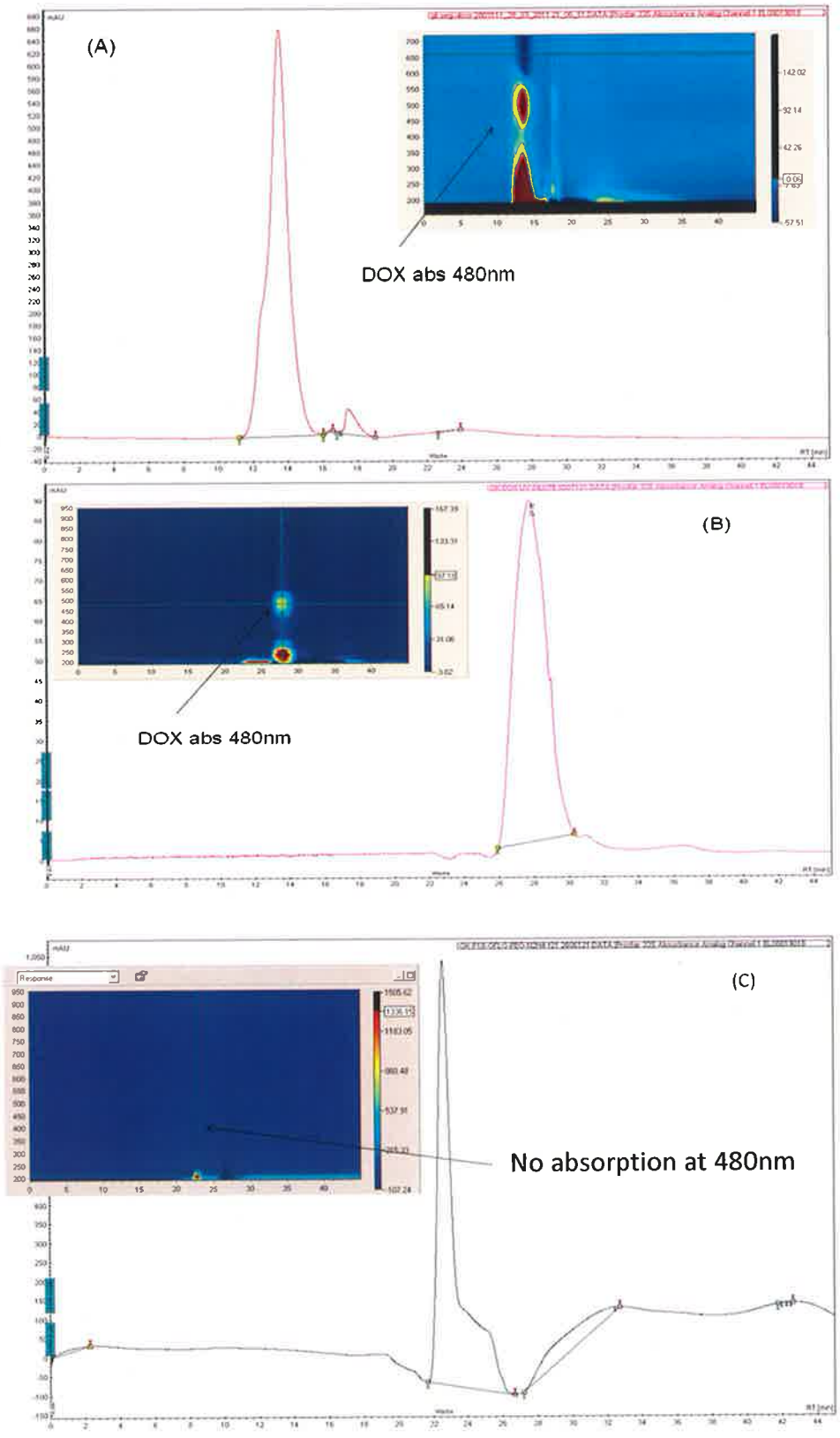
The addition of doxorubicin was performed in anhydrous methanol and in the dark, as doxorubicin is light sensitive. The reaction was monitored by SEC-HPLC and formation was observed at dual wavelengths of 214 nm for the peptide and 480 nm for doxorubicin. The formation of the hydrazone bond was clearly observed by SEC-HPLC and was monitored for 24 hours, until no further progression of the reaction could be observed. The compound was purified using sephadex LH-20 and its purity was determined by SEC-HPLC. However, MALDI-TOF mass spectrometry analysis could not be applied to this conjugate. Matrices for MALDI-TOF mass spectrometry use acidic conditions to protonate the peptides which hydrolyse the hydrazone bond linking doxorubicin to the PEGylated peptide. Several different matrices were

tested but couldn't afford a mass spectrometric analysis of the conjugate (Table 2.5).

Table 2.5: Matrix solutions used for DOX-PEG$_{2000}$-GFLG-P18 analysis

Matrix	Expected Mass	Mass Found
α- cyano 4-hydroxycinnamic acid	5476	2788.7488
2,5-Dihydroxybenozic acid	5476	2754.6643
Sinapanic acid	5476	negative
Dithranol	5476	2756.6626

The presence of the PEG in the conjugate also complicated the acquisition of its mass spectrum. MALDI spectrometers equipped with a Time of Flight detector are not well suited for acid sensitive PEGylated compounds which do not travel efficiently through a TOF detector. A higher ratio of matrix to sample is required for desorption and also the compound needs to be protonated. The presence of PEG is also problematic in LC-MS as the PEGylated compounds are not volatile enough to be vaporised at the interface. However we were able to determine the presence of DOX by UV and visible absorption analysis. This was done by careful analysis of the retention times of the purified sample by RP-HPLC which indicates florescence of the free DOX compared to the PEGylated sample. The DOX absorption, in the spectrum, is at 480nm (B) while the pure PEGylated peptide shows no absorbance in that region(C). After conjugation of the DOX, there is absorption for the DOX along with the PEGylated peptide (A). By this analysis of the absorption and retention time, the free DOX can be eliminated by SEC LH-20. From this we can conclude that the DOX is present on the PEGylated peptide.



2.37: HPLC chromatogram showing UV detection of doxorubicin: (A) PEGylated peptide with doxorubicin attached and (B) Doxorubicin (c) PEGylated peptide

Alternative analysis techniques were also attempted, including NMR. However the NMR spectra could not provide convincing evidence, as the large PEG signals dominated all other signals. Furthermore the doxorubicin signals overlapped with those of the peptide and could not be extracted unambiguously.

Although the final product of the dual-release polymeric prodrug was successfully produced, its yield, reduced by the linearity of the synthetic route and inherent low efficiently of some steps (e.g. reactions involving PEG), was insufficient to support complete biological testing. The linear approach of combining a classical cytotoxic drug, doxorubicin (DOX), and a host defence peptide (P18) to a polymeric carrier was therefore considered to be not sustainable. It was expected that simply combining two polymeric prodrugs of doxorubicin and P18 could provide the same advantages than the dual-release prodrug and afford a synergistic or additive effect, while being more synthetically accessible. This approach can also be used to alter the ratio of the peptide/DOX delivery from 1:1 (linear synthesis) to a more optimum level by a combination study.

2.4 Synthesis of polymeric prodrugs of P18 and Doxorubicin for combination studies

2.4.1 PEGylated-P18 and Synthesis of Controls

Peptides H-GFLG-P18 and H-GG-P18 were synthesised from an amide PEGA resin as described above and the resin-bound peptides were PEGylated at their N-termini with a monofunctional PEG (MeO-PEG-COOH). HATU was used to couple these PEGs and two PEGylated peptides were successfully prepared by manual synthesis, using 2 equivalents of PEG2000 or PEG5000. This conjugation was monitored by Kaiser test. The PEGylated peptides were cleaved by using normal cleavage conditions. The purification of the PEGylated peptides was performed with sephadex G-20 or G-50 depending on the size of the PEG derivative used, PEG2000 or PEG5000 respectively. The separation was monitored by analytical RP-HPLC using

a C5 Jupiter column and/or by SEC-HPLC with a BiosepS2000 column. This purification step successfully separated the free peptides from the PEGylated peptides. Chromatogram (Figure 2.38(A)) represents crude material after cleavage of the PEGylated peptide, with the peak at higher retention time representing the PEGylated peptide and the peak at lower retention time representing the free peptide. Chromatogram (Figure 2.38(B)), recorded after size-exclusion sephadex G50 and analysis by RP-HPLC C5, shows the removal of the free peptide from its PEGylated counterpart.

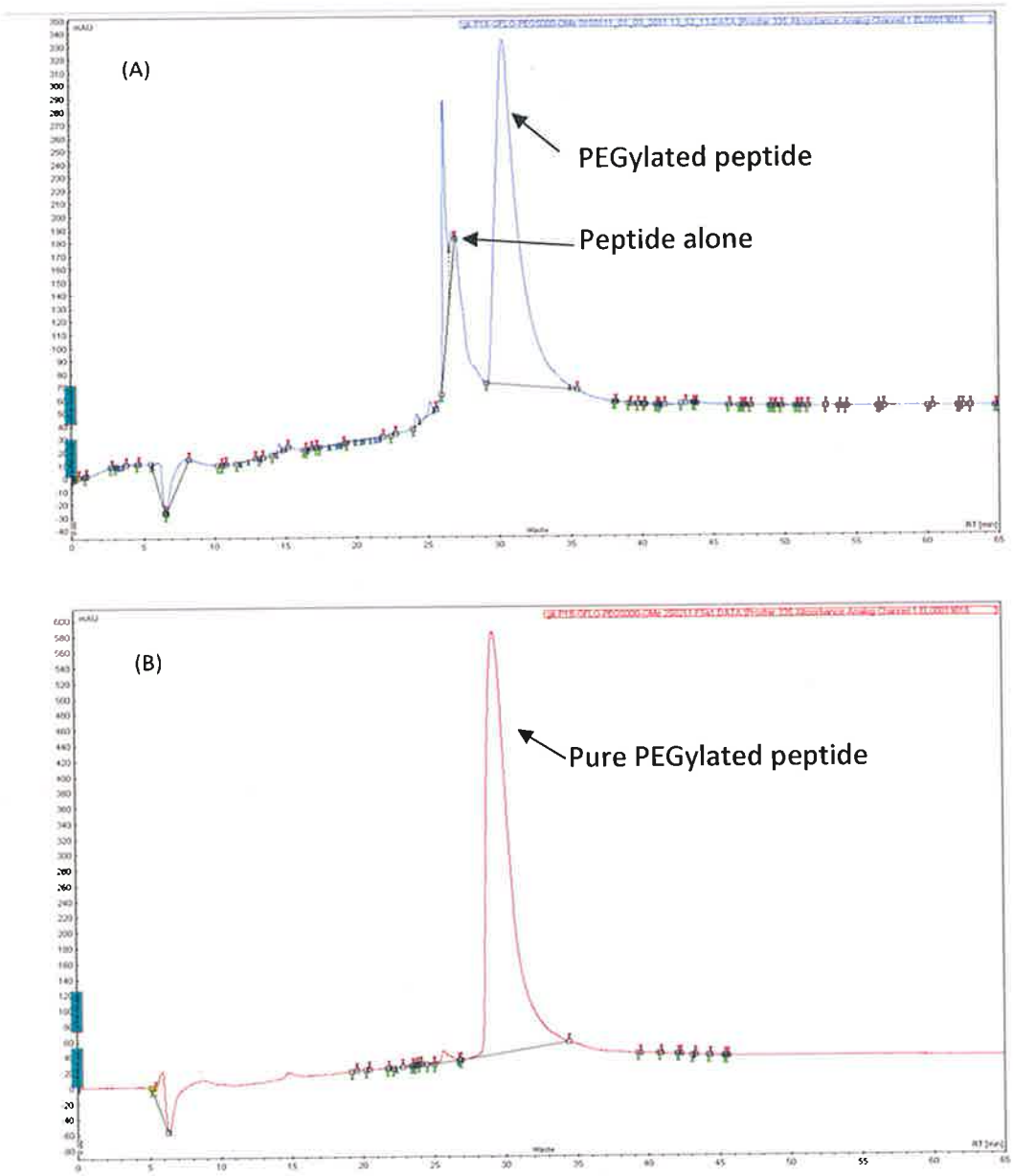


Figure 2.38: HPLC Purification of PEGylated peptide compound MeO-PEG$_{5000}$-P18-NH$_2$,

2.4.2 PEGylated Doxorubicin

PEGylated Doxorubicin was synthesised from a Rink amide PEGA resin by manual peptide synthesis. Fmoc-HN-PEG_{5000}-COOH was used with 2eq. of HATU as the coupling reagent to anchor the polymer on the resin through an amide bond. The coupling and Fmoc-deprotection reactions were monitored by the Kaiser test. *S*-Benzyl thiosuccinic acid was then added using HATU/DIEA coupling chemistry and the hydrazine group introduced as described above. Once cleaved from the resin, using 95% TFA and 5% DCM, a number of different techniques were attempted to analyse the hydrazide-PEG-amide reagent, before its conjugation to doxorubicin, as well as the PEGylated doxorubicin after conjugation, including Mass Spectrometry, NMR and HPLC. The conjugation reaction (hydrazone formation) was also monitored by SEC-HPLC, by recording the chromatograms at 480nm, a wavelength corresponding to the maximum absorption of doxorubicin. The chromatogram shown in Figure 2.39 (A), represents the reaction after 3 hours, with the first peak showing the formation of the PEGylated doxorubicin. The second peak represents the PEG-hydrazide and the third represents doxorubicin alone. Chromatogram (B), the crude compound, shows the removal of DOX by sephadex LH-20 but the sample remains a mixture of PEGylated doxorubicin and the hydrazide-PEG-amide reagent. Chromatogram (C) is the re-purification of the PEGylated doxorubicin and the hydrazide-PEG-amide compound and shows the pure PEG-Doxorubicin conjugate, while the photodiode array (PDA) detector analysis confirmed the presence of doxorubicin at the wavelength of 480nm. The presence of doxorubicin in the conjugate was also confirmed by UV-VIS spectroscopy at the same wavelengths 485.6nm and 517.4nm respectively (D).

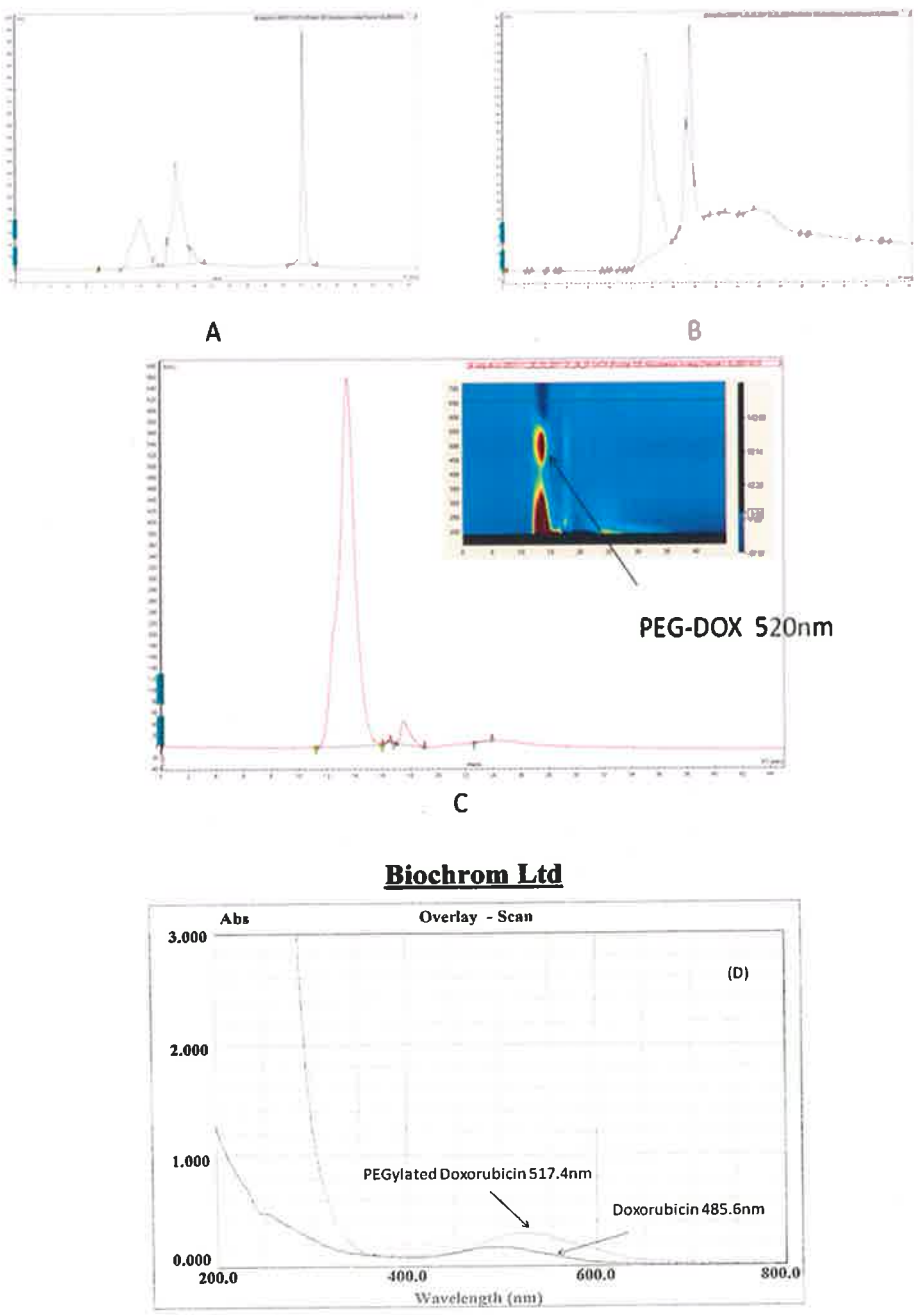


Figure 2.39: SEC chromatograms of PEGylated doxorubicin; A) after 3 hours B) crude product C) Purified PEGylated doxorubicin D) UV-vis spectrum showing the presence of DOX

The yields of these purified PEGylated peptides and doxorubicin were significantly higher than those obtained by the linear synthetic method. They were isolated in sufficient quantities for biological testing and combination studies across different cell lines. Below is a table of products and controls synthesised for testing.

Table 2.6: Compounds for cytotoxicity and combination studies

Compound	Description
P18	Peptide
MeO-PEG_{5000}-GFLG-P18	Polymeric Prodrug
MeO-PEG_{5000}-GG-P18	Negative control
MeO-PEG_{2000}-GFLG-P18	Polymeric prodrug
MeO-PEG_{2000}-GG-P18	Negative control
Cis Platin	Anticancer drug
Cis Platin	Anticancer drug

2.5 Cytotoxicity testing of PEGylated peptides and Combination study with a PEGylated classical anti cancer agent.

Cancer cell lines 4t1.2 Luc were first chosen to complete the MTS cellular assay. These mouse mammary adenocarcinoma (breast cancer) cells expressing the luciferase gene were selected for their potential application in an orthotopic mouse model, for future *in vivo* studies. This was organised as part of a collaboration with the group of Dr Judy Harmey at RCSI, as they had already established the mouse models and validated the use of the cell lines in this model. Doxorubicin is also well known for the treatment of breast cancer associated with the 4t1.2 cell line, an ideal starting point for the cellular testing of the doxorubicin compounds synthesised.

P18 was first tested for its cytotoxic activity against the 4t1.2 Luc cell line. The IC_{50} value of P18 (16μM) alone was approximately 4-fold higher than the values commonly reported in the literature for this peptide (dependent on cell line tested)[86]. Furthermore, a substantial loss of activity was observed with the polymeric prodrug candidate MeO-PEG_{5000}-GFLG-P18-NH_2, which displayed an IC_{50} value of 59 μM, a 3.6 fold loss of activity compared to the positive control P18 alone.

Further testing of these candidates was performed in collaboration with the group of Dr Siobhan McClean in the Institute of Technology at Tallaght, to include an ovarian cancer cell line SK-0V3 and an ovarian non-malignant cell line Hs832. The candidates tested against these cells and their IC_{50}'s are listed in Table 2.7 below.

Table 2.7: Cytotoxicity of the polymeric prodrugs, their active component and a peptide prodrug negative control against ovarian cell lines.

*Cell Line	P18	DOX	MeO-PEG_{5000}-GFLG-P18	MeO-PEG_{2000}-GFLG-P18	MeO-PEG_{2000}-GG-P18	PEG_{5000}-DOX
SKOV3	17.3±11.5	> 100	65.3 ± 4.1	39.4 ± 2.1	77.9 ± 8.9	>100
Hs832	5.8 ± 0.3	>100	42.3 ± 4.1	19.0 ± 2.9	51.8 ± 11.7	>100

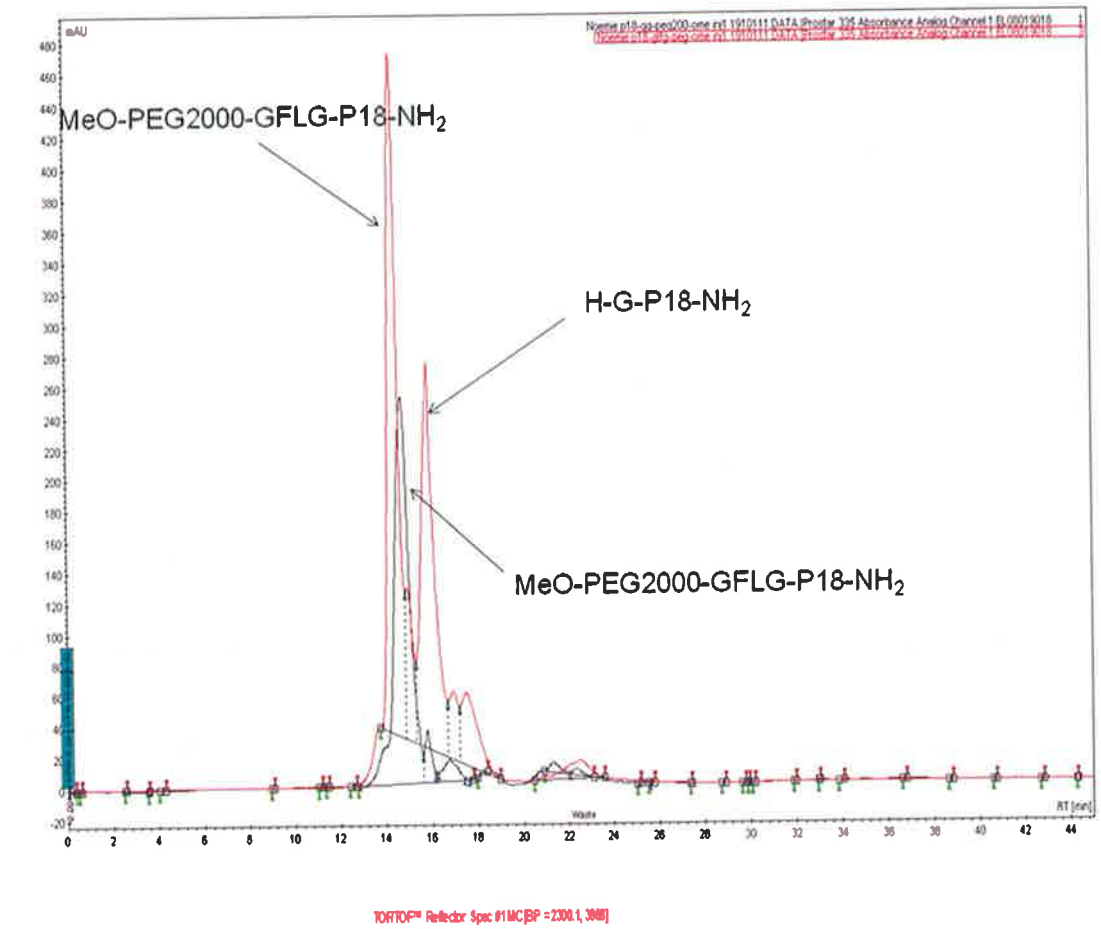
*all IC_{50}s are in μM and determined 24 hours

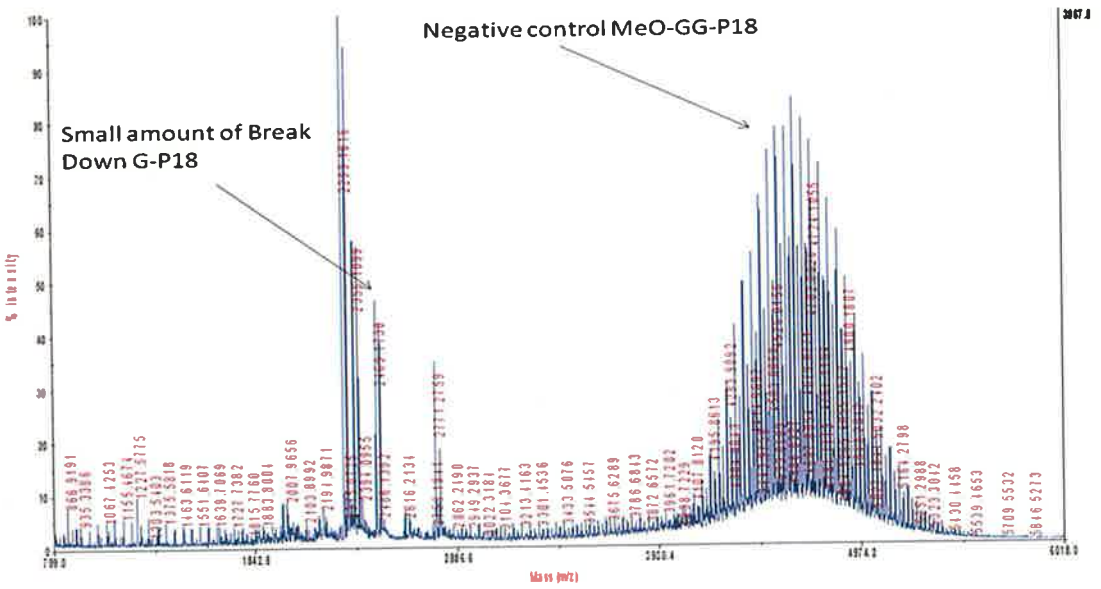
The cytotoxic activities of P18 against the SKOV3 (IC_{50} of 17.3 ± 11.5 μM) and 4t1.2 Luc cell lines were comparable, indicating that the peptide cannot promote cell death as effectively than in the cell lines previously reported in the literature. The prodrugs MeO-PEG_{5000}-GFLG-P18 and MeO-PEG_{2000}-GFLG-P18 showed a much higher activity towards the SKOV3 cell line than the negative control MeO-PEG_{2000}-GG-P18, indicating that the PEG is indeed inhibiting the activity of the P18. However, the higher IC_{50}'s of these prodrugs, compared to the peptide alone, indicate that the latter is not being fully released for the PEG. This may be due to the inefficient enzymatic release of the GFLG linker or that the PEG may be sterically hindering the cleavage site on the tetra-peptide linker and preventing its access by cathepsin B. Cancer cells do in general over-express the enzyme cathepsin B so the activities of the prodrugs should approach those of the (positive) control P18. On the other hand, the PEGylated Doxorubicin with a PEG_{5000} showed no effect against the SKOV3 cell line, which over-expresses the P-glycoprotein. The p-glycoprotein acts as a mediator of drug resistance, pumping out the doxorubicin and resulting in IC_{50}s values greater than 100μM.

An unexpected result was the relative toxicity of P18 towards the Hs832 (non-malignant) cell line with an IC_{50} of 5.8 μM. This is not consistent with the selectivity of P18 towards cancer cells previously stated in the literature[86].

The results obtained with the ovarian cell lines indicate that lower IC_{50} values are obtained with prodrugs containing a PEG_{2000} when compared to the PEG_{5000} conjugate and would suggest that shorter PEG chains generate better candidates for the enzymatic activation of the prodrug. Another proposed explanation of the results obtained with the peptide prodrugs was that the cells being studied didn't produce enough Cathepsin B to activate efficiently the prodrug candidates and/or the sequence of the linker cannot be efficiently cleaved by this enzyme. Studies were therefore conducted with purified cathepsin B enzyme to confirm that the prodrugs can be activated by this targeted enzyme.

The study was performed using commercially available cathepsin B and the MeO-PEG_{2000}-GFLG-P18-NH_2 prodrug candidate or the negative control MeO-PEG_{2000}-GG-P18-NH_2. The reaction was performed by incubating the PEGylated peptides with the enzyme for 24 hours and monitored by RP-HPLC (Figure2.40). The peptide fragments formed and identified by mass spectrometry analysis showed that linker was cleaved between the leucine and the glycine of the tetrapeptide linker, generating G-P18 (m/z=589.52 z=4) and also at MeO-PEG_{2000}-GFL-OH (Not seen by Mass Spectrometry). However, MALDI TOF analysis shows that the negative control, MeO-PEG2000-GFLG-P18-NH_2 is intact after 24hours with only small evidence of breakdown at m/z 2356.1758.





2.40: SEC-HPLC showing breakdown of MeO-PEG$_{2000}$-GFLG-P18-NH2 and MALDI TOF analysis of negative control intact MeO-PEG$_{2000}$-GG-P18-NH$_2$

Following the results of this assay with purified cathepsin B, human ovarian cancer cells A2780P were chosen as a suitable target for the evaluation of the peptide prodrugs. A2780P cells are known to express high levels of cathepsin B. Furthermore these cells are sensitive to doxorubicin in the nanomolar range. Table 2.8 below shows the results of the peptide prodrugs and their analogues against this cell line.

Table 2.8: Compounds tested against A2780P

Cell Line	P18	DOX	MeO-PEG_{2000}-GFLG-P18	MeO-PEG_{5000}-GG-P18	PEG_{5000}-DOX	MeO-PEG_{2000}-GFLG-P18 + PEG_{5000}-DOX
A2780P	2.7	98 nM	6.8	87	29	2.93

all IC_{50}s are in μM unless stated MTS assay at 72 h

P18 shows a 5.9 fold increase in activity towards the A2780P 2.7 μM cells compared to the 4t1.2 Luc breast cancer cell lines (16 μM). This is comparable to the level of activity reported in the literature for this peptide. The IC_{50} value of the prodrug candidate MeO-PEG_{2000}-GFLG-P18-NH_2 was 6.8 μM. There is therefore a 2.5 fold activity differential with the P18 itself, but this differential is significantly less than for the negative control MeO-PEG_{2000}-GG-P18-NH_2 which has an IC_{50} value of 87 μM. There is therefore a 32 fold difference in activities between the active peptide and the negative control prodrug. These results which are consistent with a prodrug behaviour of the MeO-PEG_{2000}-GFLG-P18-NH_2 candidate can be explained by the higher expression of cathepsin B in A2780P cells, compared to the 4T1.2 Luc and SKOV3 cancer cell lines.

The PEGylated prodrug of doxorubicin showed a 290 fold decrease in activity with an IC_{50} value of 29 μM, compared to doxorubicin alone (98 nM). The PEG_{5000}-DOX-NH_2 contains an acid labile bond and requires a change in pH in order for the DOX to be released from the PEG. This is proposed to happen in the lysosome of the cell. However it is possible that the PEG5000 is too large, encapsulating doxorubicin and

not allowing its full release from the PEG. The PEG is capable of attracting up to 2-3 water molecules per PEG unit so it is probable that the acid labile bond is staying intact due to the large swelling hydrophilic properties of the PEG.

However there is still some activity restored from the PEG_{5000}-DOX, at a sufficient level to warrant further investigation in combination studies between the PEG_{5000}-DOX and the MeO-PEG_{2000}-GFLG-P18-NH_2. The investigation aims at establishing if the combination of the 2 prodrugs can generate synergistic/additive effects. The results were therefore expressed as the fractional inhibitory concentration (FIC) index, which is assased as follows:

FIC= [A]/IC50 Value A + [B]/IC50 Value B

Where IC_{50} value A and IC_{50} Value B are the IC_{50} values of the compounds alone and [A] and [B] are the IC_{50} Values when they are in combination. An FIC index of 0.5 is taken to indicate good synergy while an FIC index of 1.0 represents additive activity. The two compounds were tested against the A2780P cell line and the values obtained were found to be synergistic with IC_{50} values of 2.93 μM for the MeO-PEG_{2000}–GFLG-P18-NH_2 and MeO-PEG_{5000}-NH_2 with an FIC value of 0.65 indicating a synergistic effect.

2.6 Summary

Polymeric peptide prodrugs and their analogues were synthesised by solid phase peptide synthesis. The first approach based on a linear synthetic method could be successfully developed, but because of the linear multistep synthetic approach and a complicated purification process, it was found to be not economically sustainable while being also limited to the delivery of doxorubicin and P18 in a 1:1 ratio. The combination of 2 separate polymeric prodrugs became more appealing from a chemical synthesis and a biological perspective with the potential to optimise the ratio between the peptide and the doxorubicin. The co-delivery of these 2 agents to solid tumours can be achieved through the EPR effect.

The active peptide agent P18 showed a range of different activities against different cancer and non-cancerous cell lines. The 4T1.2 Luc and the SKOV3 cell lines showed a relatively low sensitivity to this candidate. In addition, the SKOV3 cells were also resistant to doxorubicin through the up regulation of P-glycoprotein. A surprising result was the greater toxicity of P18 towards the non-malignant cell line Hs832, compared to their malignant counterpart. Further studies with these malignant and non-malignant ovarian cells would be of interest.

The A2780P cell line was more susceptible to P18, with had activities against this cell line in the low μM range. The results obtained with the peptide, peptide candidates and negative prodrug control indicate that PEG_{2000} can mask the activity of P18 and that activation of P18 through cleavage of the tetra-peptide linker occurs. The PEGylated prodrug of doxorubicin on the other hand does not appear to allow efficient activation of doxorubicin, potentially because of the large size of the PEG used, which can inhibit the access of the surrounding liposomal environment to the hydrazone bond. The combination of these two polymeric prodrugs did show a synergistic effect which is promising and would warrant further investigation with a smaller PEG chain.

Chapter 3:

Oligoglutamic acid peptide prodrugs

3 Overview

Cationic Peptides have a binding selectivity for cancer cells due to their net positive charge and amphipathic nature. The free peptide P18 has activity in the low μM range on various different cancer cell lines[85]. Although this peptide is selective for cancer cells through essentially electrostatic interactions, it can also be toxic against normal mammalian cells at the concentration levels required to maintain therapeutic levels *in vivo*[59b]. As the net charge of a peptide is a critical determinant of its activity, a peptide prodrug of the highly charged P18 was designed by reversibly reducing its net +8 charge. As discussed in the previous chapter, enzymatic activation is a valuable tool for the selective release of peptides and/or other drug candidates in a catalytic manner. Enzymes uniquely or over-expressed in cancer cells represent attractive targets to confine the activity of a toxic agent modified as a prodrug to tumour sites. Using this technique is a way of controlling drug release and overcoming toxicity issues of drug candidates and/or peptide drugs alone[90]. In the case of cationic peptides such as P18, the net positive charge can be modulated through the reversible conjugation to negatively charged amino acids such as aspartic and glutamic acids to form the peptide prodrug. As aspartic acid is associated with multiple synthetic and stability problems, glutamic acid was selected to counterbalance the cationic lysine residues of P18. Enzymatic site specific release within the cancer cell of these glutamic acids from the parent peptide would allow its targeted delivery to cancer cells[134]. The focus of this chapter will be the use of proteolytic protease enzyme activation mechanisms.

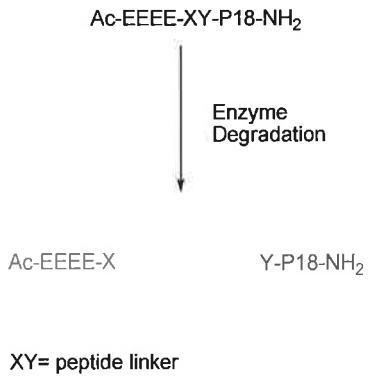


Figure 3.1: Peptide Prodrug release of P18

There are a significant number of up-regulated proteolytic enzymes found in cancer cells compared to normal cells which can be exploited for site specific activation of anticancer prodrugs[149]. Along with cathepsin B, which was used previously for the polymeric prodrugs of the same peptide candidate, another enzyme that has been identified by Dr Warren Thomas from the Department of Molecular Medicine at RCSI, is Matrix Metalloproteinase -2 (MMP-2). MMP-2 is an enzyme that is found to be over produced in particular in malignant mesothelioma[150]. These specific targeting enzymes are found to be more effective and more reliable than other mechanisms such as chemical activation mechanisms involving acid labile bonds. By introducing enzyme sensitive peptide linkers in between a cationic active sequence and an anionic promoiety, reversible masking of the peptide's net charge, controlled by cancer cells, can be achieved.

3.1.1 Cathepsin B

As mentioned previously, Cathepsin B is also one the main targets of these prodrugs as it is considered to be one of the best intracellular proteases. It is highly unregulated in malignant tumours[151]. Cathepsin B is specific to a number of different amino acid combinations but with most effective is Gly-Phe-Leu-Gly where it has been proven to be the most effective and containing plasma stability and

rapid hydrolysis in the presence of cathepsin B and therefore many prodrugs are based around the tetrapeptide[152]. In chapter 2, Cathepsin B was used in combination with the polymeric peptide conjugate P18 and the delivery of the anticancer drug doxorubicin. In this chapter we will also focus on the enzymatic cleavage of the anticancer peptide P18 in the form of a prodrug.

3.1.2 Metalloproteinases

Inhibition of Matrix Metalloproteinases (MMPs), a family of zinc- and calcium-dependent peptidases involved in the regulation of cellular behaviour by proteolytic processing of the extracellular environment, has long been proposed as a novel therapeutic strategy for the treatment of diseases like cancer and arthritis[149]. In cancer, malignant tumours invade normal tissue, a process that involves three independent processes: the degradation of the extracellular matrix (ECM), cell migration, and proliferation. Human pleural malignant mesothelioma is a malignancy that is characterized by aggressive local spreading into the pleura and the surrounding tissues, but has a low rate of distant metastasis. Malignant invasion of tissue is a dynamic, complex, and multi-step process in which the proteolytic degradation of the basal membrane ECM is an essential step. ECM proteolysis depends on the action of the cysteine-, serine- aspartyl-proteinases, and of the matrix metalloproteinases (MMPs). In particular, the gelantinases, MMP-2 and MMP-9 are capable of degrading most ECM components that form the basal membrane making them essential for tumour cell migration, tumour spreading, tissue invasion of tumour cells and metastasis[150]. However, MMP-2 is not only expressed by the pleural malignant mesothelioma cells but also by surrounding stroma cells[153]. Work carried by Chau and collaborators has shown that oligopeptides that are sensitive to the enzymatic cleavage by MMPs may be added to synthetic polymers and peptides-amphiphiles[154]. Their work describes the insertion of a cleavable peptide sequence constituting a substrate for MMP-2 (PVGLIG) into a self-assembled peptide sequence for eliciting cell and tissue remodelling activities. Incorporating MMP-cleavable substrates is an attractive

strategy to engineer controlled release of a peptide from its prodrug within the mesothelioma cell.

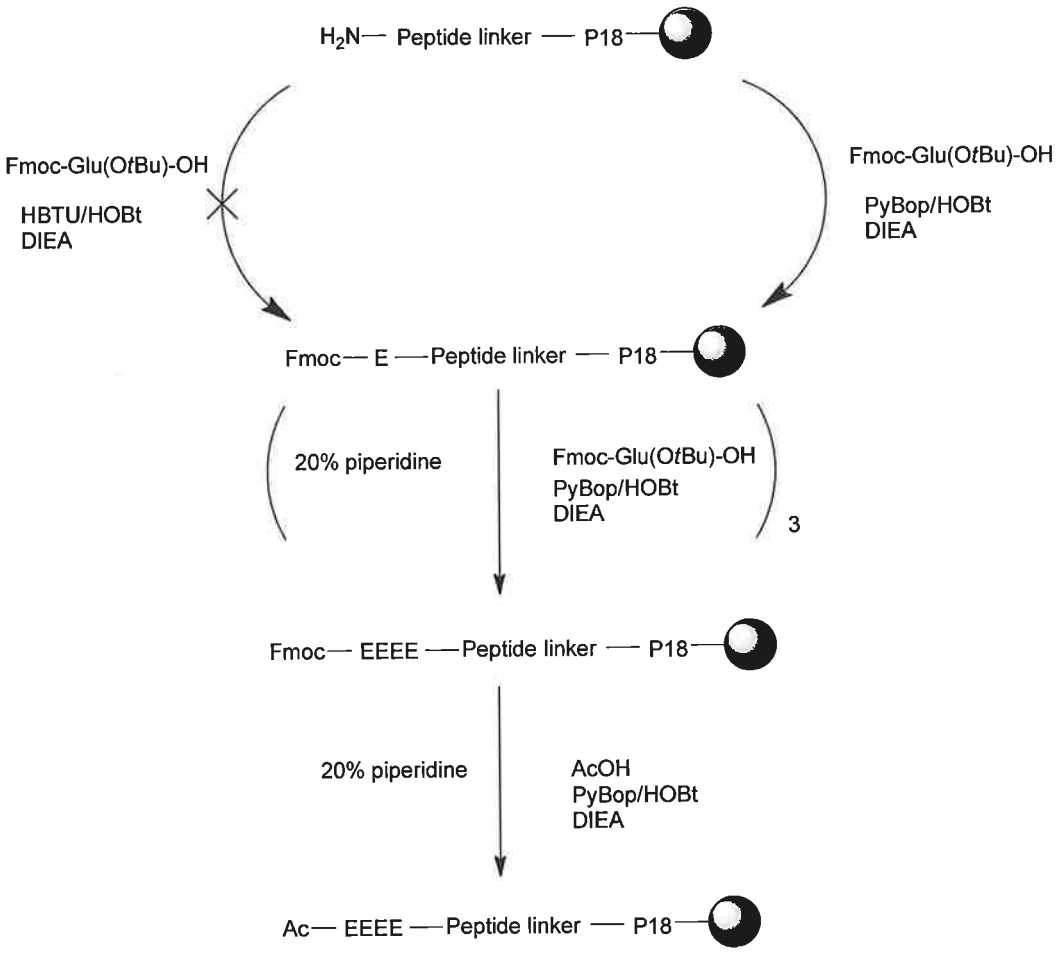
3.2 Malignant mesothelioma

Malignant mesothelioma is an aggressive and invariably fatal serosal tumour which is highly associated with the exposure to asbestos. From the time of diagnosis, median survival rate is less than 6 months. Malignant mesothelioma responds poorly to conventional modes of therapy including surgery, chemotherapy and radiotherapy[155]. Eighty percent of mesotheliomas originate in the pleural space, and they represent the most common primary tumour of the pleural cavity. Despite its relative rarity, mesothelioma remains an area of special interest in pulmonary medicine because of its increasing frequency, dismal prognosis, and attendant medicolegal issues related to asbestos exposure. Mesotheliomas are classified into three general categories: diffuse malignant, localized benign, and localized malignant. The so-called "benign mesotheliomas" are usually localised, and they have been reclassified as benign fibrous tumors of the pleura, probably arising from a cell of origin other than the mesothelium[156]. Because of the tumour's characteristically poor response to treatment this makes it an ideal target for the use of new techniques and approaches. MMPs are well known to be up-regulated in these types of tumours[150]. This makes the MMP degradable linkers a useful tool for the delivery of prodrugs.

3.3 Synthesis of peptide prodrugs

The peptides H-GFLG-P18-NH_2, H-PVGILG-P18-NH_2 and control peptide H-GG-P18-NH_2 were synthesised from a Rink amide MBHA resin as described in Compounds 5.2.1.8, 5.2.1.9, 5.4.1.3 respectively. The tetra glutamic acid motif was elongated from the peptides by manual synthesis. Guanidinium coupling chemistry using HBTU/HOBt/DIEA was first used for the coupling with up to 10 equivalents and a

double coupling procedure. However due to the increasing hydrophobicity of the peptide sequence, it was clearly shown by the Kaiser test that the coupling and deprotection steps were becoming inefficient after the addition of the second glutamic acid[157]. The coupling chemistry was changed to a phosphonium reagent, using PyBop/HOBt/DIEA and the coupling steps were successfully delivered with 5 equivalents of phosphonium reagent and hydroxybenzotriazole additive and 10 equivalents of tertiary amine. These results are consistent with previous reports indicating that PyBop is an efficient coupling reagent for sequences containing hydrophobic regions (Figure 3.2).[158]



E= Glu
peptide linker = GFLG, GG, PVGILG

Figure 3.2: Synthesis of Ac-EEEE-Peptide linker-P18-NH2

PyBop is a derivative to Bop developed by Castro and collaborators in the 1950's. One of the first representatives of generations of highly efficient coupling reagents, Bop was developed due its efficient coupling and speed of the reaction, avoiding racemization[159]. Although Bop is very efficient, PyBop was developed to avoid the carcinogenic side-product HMPA. PyBop is a coupling reagent that operates *in situ* and can be used when coupling becomes more difficult. It reacts quickly as it is only found in the guanidinium N' form unlike HBTU which is found mainly in the uronium O' Form[160]. HOBt is also used as an additive in combination with PyBop. It is slightly less efficient than HATU but is a lot cheaper and therefore can be used in higher equivalents with double or triple coupling procedures to force the reaction to completion.

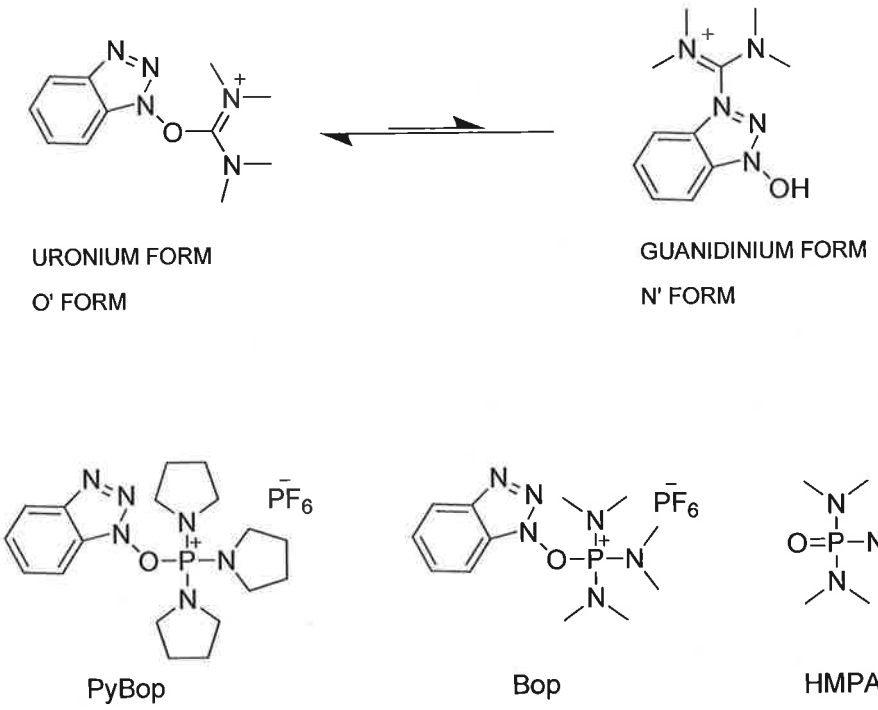


Figure 3.3: Guanidinium and uronium forms of HOBt, PyBop, Bop coupling reagents and HMPA side product

The N-terminus of the peptide was finally acetylated using 10 equivalents of acetic acid and again PyBop/HOBt/DIEA coupling chemistry. The peptides were then cleaved from the resin and purified by RP-HPLC. The following peptides and controls where synthesized and the biologically tested as described thereafter (Table 3.1).

Table 3.1: Peptide prodrugs and controls tested against various different cancer cell lines

		Cell line testing			
Peptide	Enzyme for activation	A2780P	REN	Msto-211H	H2052
Ac-EEEE-GFLG-P18- NH_2	Cathepsin B	✓	×	×	×
Ac-EEEE-GG-P18- NH_2	Cathepsin B/MMP2	✓	✓	✓	✓
Ac-EEEE-PVGILG-P18- NH_2	MMP2	×	✓	✓	✓

3.4 Biological testing

3.4.1 Growth Inhibition of A2780P ovarian cancer cell line

The A2780P cell line was used for the biological testing of the cathepsin B-dependent prodrug and its active P18 counterpart. As discussed previously, Cathepsin B is up regulated in this cancer cell line and Cathepsin B-dependent polymeric prodrugs of P18 were successfully used against these cells. The prodrug candidate Ac-EEEE-GFLG-P18-NH_2 was tested in MTS toxicity assays, along its negative control, Ac-EEEE-GG-P18-NH_2 which contains a cathepsin B-resistant glycylglycine linker.

The prodrug candidate Ac-EEEE-GFLG-P18 showed activity against the A2780P cell line with an IC_{50} of 16.9 μM. The control peptide Ac-EEEE-GG-P18 confirmed that a loss of activity is observed with this cathepsin B-insensitive linker and indicates that the removal of the promoiety from the prodrug contributes to the activity of this candidate. These results are presented in Figure 3.4.

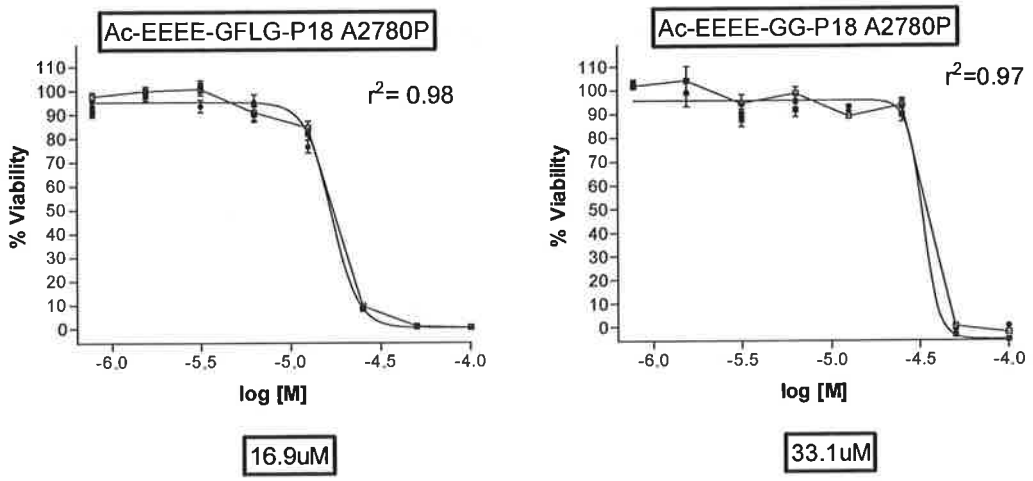


Figure 3.4: IC_{50} of A2780P against Ac-EEEE-GFLG-P18 and negative control AC-EEEE-GG-P18

However, a 2-fold decrease in activity only between the prodrug candidate and the negative control is observed and the IC_{50} of the parent P18 alone at 2.2μM is significantly lower than the IC_{50} of the prodrug, against this A2780P cell line (Figure 3.5). This could be due to am incomplete activation of the prodrug and/or to sub-optimal kinetics of activation, or even an insufficient escape of the activated peptide from the lysosome.

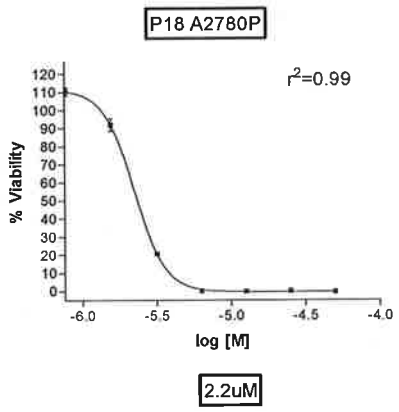


Figure 3.5: IC_{50} of P18 against A2780P ovarian cancer cell line

3.5 Biological testing of GFLG linker against mesothelioma cell lines, REN, MESO and H2052

The three cells lines REN (epithelioid-type mesothelioma), Msto-211H (biphasic-type mesothelioma) and H2052 (sarcomatoid-type mesothelioma), the most aggressive cell line type of the three where tested against the following compounds in the Education and Research Centre of the Royal College of Surgeons in Ireland, by the group of Dr. Warren Thomas (Beaumont Hospital).

Table 3.2 : Peptides and peptides prodrugs testing against different cell lines

	Cell Line		
Peptide	REN	Msto	H2052
P18	×	×	✓
Ac-EEEE-GFLG-P18	×	✓	×
Ac-GG-P18	×	✓	×
Ac-PVGLIG-P18	✓	✓	✓

3.5.1 Growth Inhibition of GFLG prodrug linker, on H2052 malignant mesothelioma cell line

P18, as the positive control has, at 24 hours, an IC_{50} of 2.5μM against the H2052 cells, showing a significant activity, for a HDP, against this most aggressive mesothelioma cell line, as presented in Figure 3.6. The negative control Ac-EEEE-GG-P18 was tested next on the H2052 cell line, and indicates cell death at 44.9 μM, as shown in Figure 3.6. This indicates nearly a 20-fold differential in the activity of P18 through the addition of an oligo-glutamic acid promoiety. The cathepsin B-dependent prodrug candidate itself, Ac-EEEE-GFLG-P18, had an IC_{50} value of 14.9μM against this aggressive phenotype, as presented in Figure 3.6.

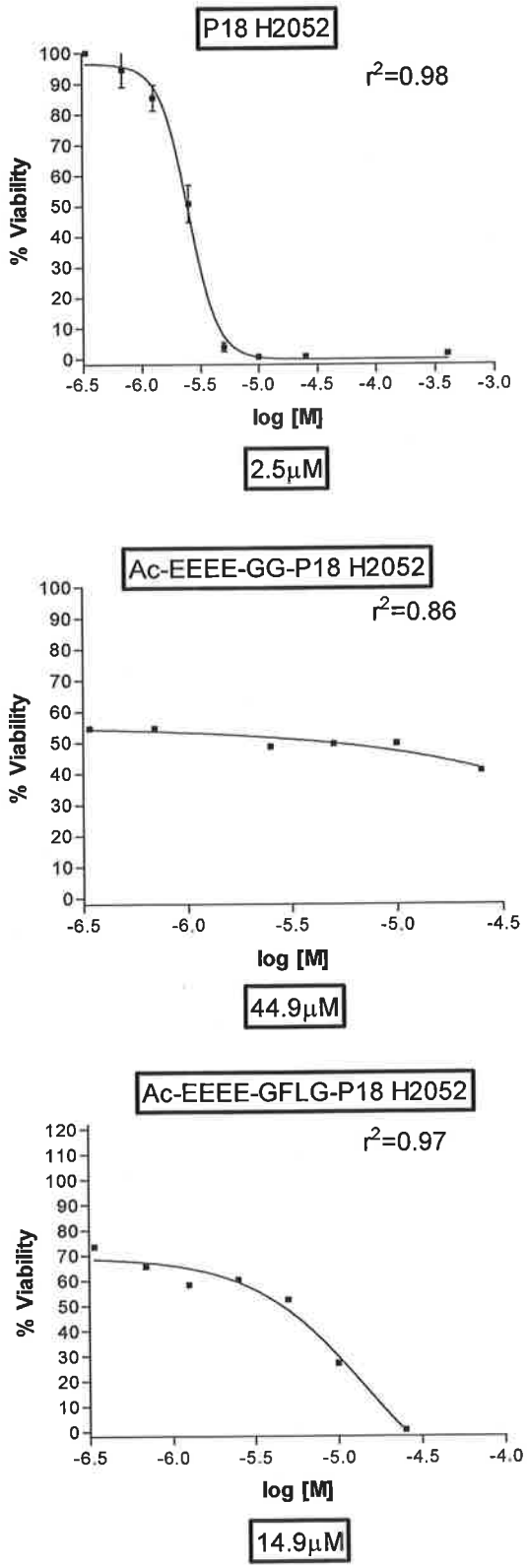


Figure 3.6: IC_{50} of P18, Ac-EEEE-GG-P18 and Ac-EEEE-GFLG-P18 against H2052 mesothelioma cell line

Full activation of the prodrug fails again to occur with these cells, with activity differentials between the active peptide, the prodrug and the negative control being even more marked. Nevertheless, the prodrug remains slightly more active against the mesothelioma cell line than the ovarian cancer cell line, despite equivalent activities of the parent peptide against these cells. These results are presented in Figure 3.7 and suggest that a slightly higher level of cathepsin B is present in the mesothelioma cell line than the A2790P ovarian cancer cell line.

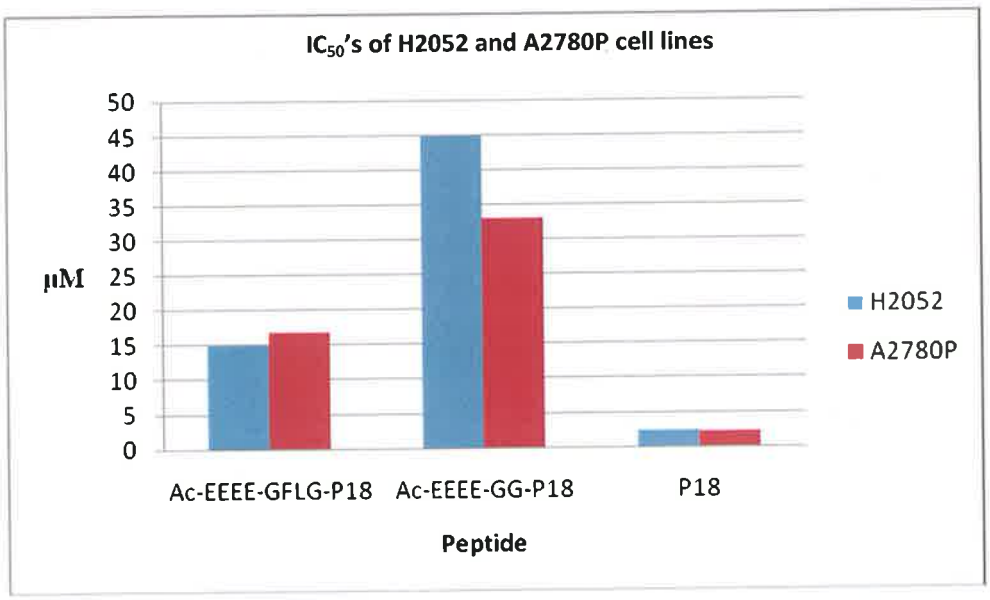


Figure 3.7: IC_{50} of Ac-EEEE-GFLG-P18, AC-EEEE-GG-P18 and P18 against mesothelioma and ovarian cancer cell lines

3.5.2 Growth Inhibition of PVGLIG prodrug linker on REN malignant mesothelioma cell line

Malignant mesothelioma highly over expresses the protease MMP-2. The prodrug candidate Ac-EEEE-PVGLIG-P18 was designed to target the high levels of MMP-2 and its ability to cleave the linker PVGLIG effectively. This candidate was tested against the 3 mesothelioma cell lines and its activities compared against P18 as a positive control. The two graphs below shows that P18 and its MMP-2-dependent

prodrug Ac-EEEE-PVGLIG-18 had IC_{50}s of 2.6μM and 0.94, respectively after 48 hours against the REN cell line, Figure 3.8.

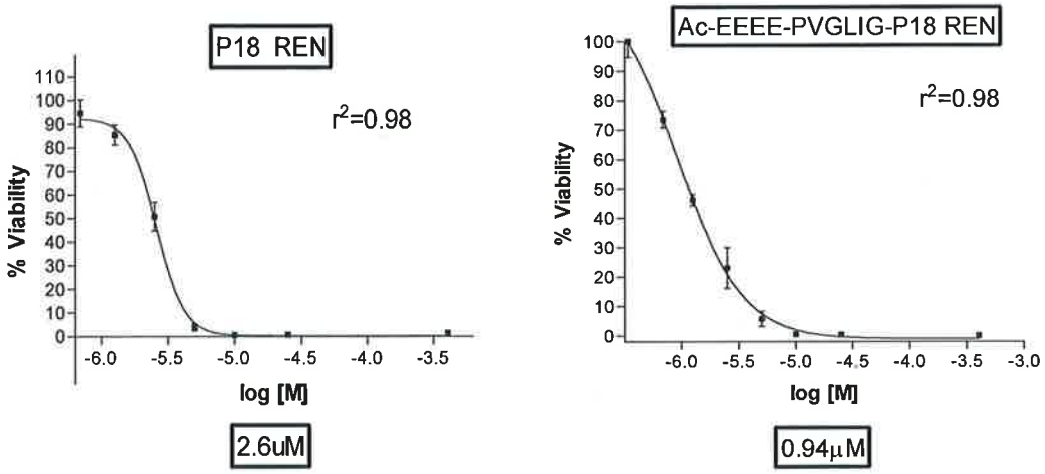


Figure 3.8: IC_{50} of P18 and Ac-EEEE-PVGLIG-P18 against REN mesothelioma cell line

In this case a 2.7 fold differential activity in favour of the prodrug is observed. This would suggest that either the intact prodrug is more active than its active peptide counterpart against these cells, or that the peptide actually released from the prodrug is more active than the native P18 itself. This can be seen on the chart below (Figure 3.9)

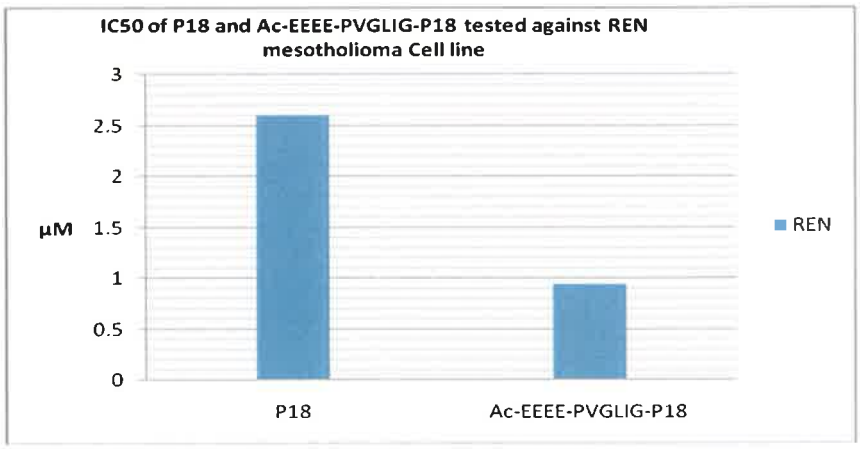


Figure 3.9: IC_{50} of P18 and Ac-PVGLIG-P18 against REN mesothelioma cell line

3.5.3 Growth Inhibition of MMP2-dependent prodrug on Msto malignant mesothelioma cell line

P18 and its MMP2-dependent prodrug (Ac-EEEE-PVGLIG-P18) were tested against the Msto malignant mesothelioma cell line. P18 had an IC_{50} of 1.44μM and the Ac-EEEE-PVGLIG-P18 had an IC_{50} of 1.33 μM. This suggests that there is full re-establishment of the activity of the P18 with a slight added increase in activity,Figure 3.10.

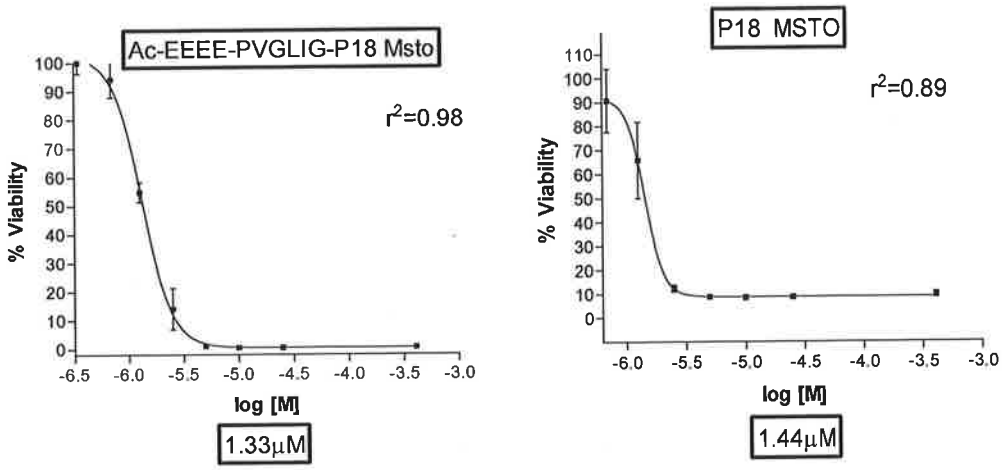


Figure 3.10: Growth inhibition of P18 and Ac-EEEE-PVGLIG-P18 against Msto mesothelioma cell line

3.5.4 Growth Inhibition of MMP2-dependent prodrug on H2052 malignant mesothelioma cell line

The H2052 mesothelioma cell line was also tested and the results obtained showed that activities of 2.5μM and 1.76μM were achieved against this cell line with P18 and its MMP2-dependent prodrug, respectively. Again, an increased cytotoxic activity is obtained from the prodrug, as compared to the P18 alone (Figure 3.11).

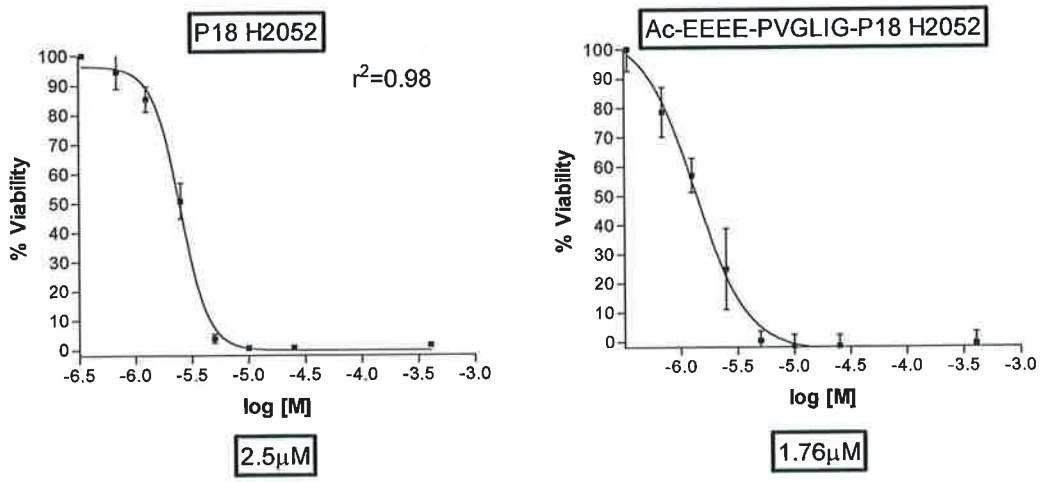


Figure 3.11: IC_{50} of P18 and Ac-EEEE-PVGLIG-P18 against H2052 mesothelioma cell line

3.6 Conclusion

P18 and its MMP2-dependent prodrug Ac-EEEE-PVGLIG-P18 were tested against three different mesothelioma cell lines. The results which are summarised below indicate that the entity released from the prodrug has a greater activity, albeit not significantly different, than the P18 parent sequence.Figure 3.12.

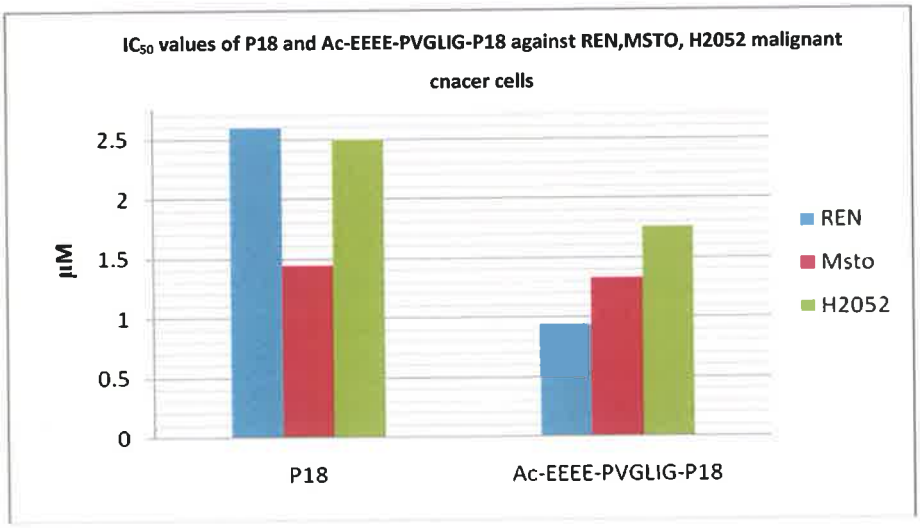


Figure 3.12: IC_{50}s of P18 and Ac-EEEE-PVGLIG-P18 against REN, Msto and H2052 malignant cancer cell lines

The reason behind this difference could be that MMP-2 processing of the prodrug leaves three residual amino acids from the linker on the released peptide. The

activation of the prodrug releases indeed the promoiety Ac-EEEE-PVG-OH and the peptide H-LIG-P18. The additional three amino acids on the P18 sequence can increase the alpha-helical secondary structure content of the peptide and therefore allow for better peptide aggregation on the cell surface which in turn causes the cells to lyse more efficently[161]. This surprising result from a prodrug approach could therefore originate from this difference between the P18 sequence and the species activated from its MMP2-dependent prodrug. This can be seen in comparsion to the Cathepsin B–dependent prodrug where the IC_{50} values had around a 4-fold difference across the 4 cell lines tested compared to the native P18. These results indicate that MMP-2 is a better target in mesothelioma for the application of antimicrobial peptide-based anticancer prodrugs. The results obtained with the GG linker contol as a negative control for the prodrug, associated with the lowest with an IC_{50} of 33μM against the A2780P cell line, indicate that the activities observed originate in the protease activation of the prodrug. Future work to be carried on the project would be to synthesize the positive H-LIG-P18 and test its activity compared to that of the prodrug.

Chapter 4:

The Synthesis of a dual acting heterodimer peptide

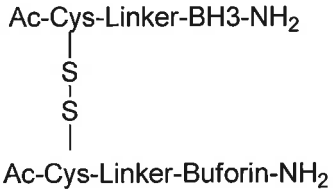
4 Synthesis of a Dual acting heterodimer peptide

4.1 Summary

Sequences of pro- and anti-apoptotic proteins can display the activity of their parent proteins. Synthetic peptides derived from pro-apoptotic proteins can therefore potentially be used as anticancer drugs[162]. The BH3 domain of Bid, for example, has the ability to enhance the apoptotic response of cancer cells to chemotherapy but unfortunately is susceptible to *in vivo* instability and is unable to translocate the cell membranes[163]. To date, to enhance the cellular uptake of this peptide, permanent modifications of the sequence with fatty acids or cell-penetrating peptides have been performed. Host defence peptides, such as buforin, are molecular mediators of innate immunity in multi-cellular organisms. The ability of some of these peptides to induce mitochondria-dependent apoptosis in cancer cells, to overcome drug resistance mechanisms affecting classical anti neoplastic agents, combined with their low propensity to select resistant mutants and activity against both dividing and non-dividing cells, have made them attractive anti-cancer leads[164]. Interestingly, these peptides have also the ability to translocate cellular membranes and promote the uptake of a conjugated moiety[165]. However, their capacity to act as vectors has not been exploited to date. This makes buforin an excellent candidate not only as a carrier for a BH3 peptide, but also as an anticancer candidate in its own right.

4.2 Aim of the project

The aim of this project is to synthesise a heterodimeric peptide including the BH3 domain of the pro-apoptotic protein Bid and the buforin sequence, as represented in Figure 4.1. Both sequences can be synthesised with a cysteine residue at their N-termini to cross-link them through a disulfide bond.



linker = β-Alanine, Hexanoic acid

Figure 4.1: Heterodimer peptide including the BH3 domain sequence of Bid and a host defence peptide buforin IIb

They are both amenable to assembly by Solid Phase Peptide Synthesis (SPPS) according to the Fmoc/*t*-Bu strategy. As the activity of membrane active peptides is independent of the stereochemistry of their constitutive residues, the buforin sequence can be assembled from D- amino acids, while the BH3 sequence has to be assembled from natural L- amino acids[163]. The use of a cross-linking reagent (2,2'-dithiol-bis(5-nitropyridine), DTNP) was chosen to favour the formation of the heterodimer over their homodimeric counterparts[166]. Conjugating the two peptides via a disulfide bond allows the BH3 peptide to be released from the buforin sequence by exploiting the reducing conditions inside cells, after buforin- mediated cellular uptake[167]. Ultimately, formation of 2 disulfide bonds between the two peptide candidates, located at their N- and C-termini, will be performed, to impart increased metabolic stability to the BH3 peptide candidate, the all-D buforin sequence being already proteolytically resistant. Formation of the (N-terminal) disulfide bond will be performed as outlined above, while macrocyclisation to the heterodetic peptide could be performed by deprotection of C-terminal acetamidomethyl-protected cysteines and disulfide bond formation[166]. This conjugate and the buforin peptide alone will be used to compare the activities of the heterodimer to those of its parent peptides used as single agents.

4.3 The apoptotic signalling pathway

Cancer cells show deviant behaviour that involves apoptotic signalling. In order for cancer cells to survive, cancer cells typically undergo changes which allow them to evade cell death signals[19]. A family of proteins known as caspases are characteristically activated in the early stage of apoptosis. Caspases are the essential component of the apoptotic process which leads to cell death. These proteins break down or cleave key cellular components that are required for normal cellular function. There are 3 major pathways associated with caspase activation, the extrinsic or death receptor pathway, the intrinsic or apoptosome pathway and the cytotoxic lymphocyte granzyme B pathway[168], as presented in Figure 4.2. The intrinsic pathway is triggered in response to a broad range of death stimuli that are generated from within the cell, such as DNA damage[168-169]. The inactivation of this pathway is normally regarded as a trademark of cancer. The intrinsic pathway is mediated by mitochondria, and, in response from apoptotic stimuli, numerous proteins are released from the intermembrane space of the mitochondria into the cytoplasm[168]. The pro-apoptotic protein, cyctochrome c is able to bind and activate the protein APAF1 in the cytoplasm, eventually leading to a cascade of caspase activation[66]. The extrinsic pathway is initiated by the binding of an extracellular death ligand to its cell surface death receptor. The extrinsic pathway can crosstalk to the intrinsic pathway through the caspase- 8-mediated cleavage of BID, a BH3 only protein BCL2 family member which triggers the release of mitochondrial proteins[170].

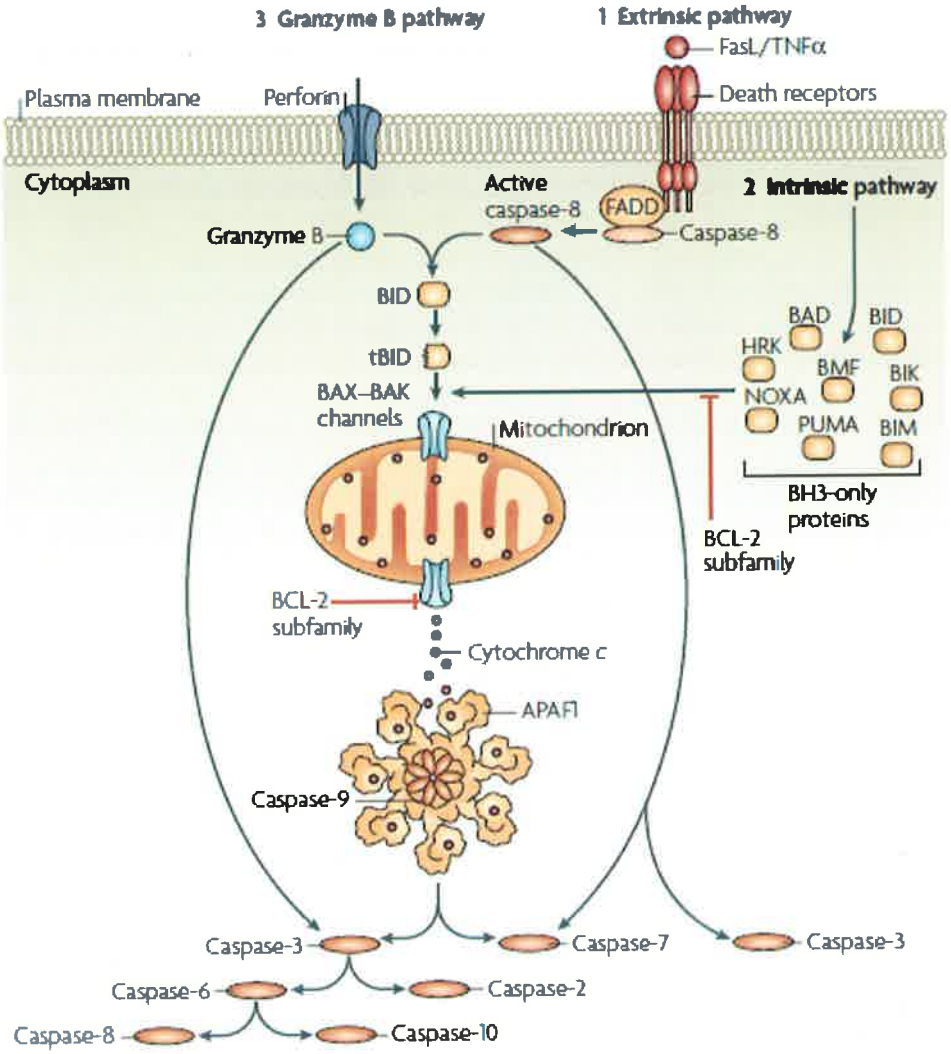


Figure 4.2: Extrinsic, intrinsic and granzyme pathways; Figure taken from Nature Reviews molecular cell biology, Vol 9, March 2008, 233

4.4 Role of B-Cell lymphoma-2 (BCL-2) proteins

BCL-2 family proteins have a crucial role in the regulation of apoptosis through their ability to regulate mitochondrial cytochrome *c* release which results in a series of caspase activation and causes cell death[171]. Bcl2 family proteins regulate apoptosis along the intrinsic mitochondrial apoptosis pathway that is activated in response to a number of stress stimuli including growth-factor deprivation, cytokine-withdrawal, Ca^{2+}-flux or DNA-damage, caused by UV or gamma irradiation, but can also be licensed to contribute to cell death triggered by members of the tumour

necrosis factor family member such as Fas, TNF or TRAIL[169]. The BCL-2 family comprises of both anti- and pro-apoptotic molecules and these are broken down into three sub-families based on their homology domains and function. They contain between one and four BCL-2 homology (BH) domains, as shown in Figure 4.3. The anti-apoptotic subfamily comprises proteins that contain four BH domains. Most members of this subfamily also contain transmembrane domains (TM) and are therefore typically associated with the membrane.

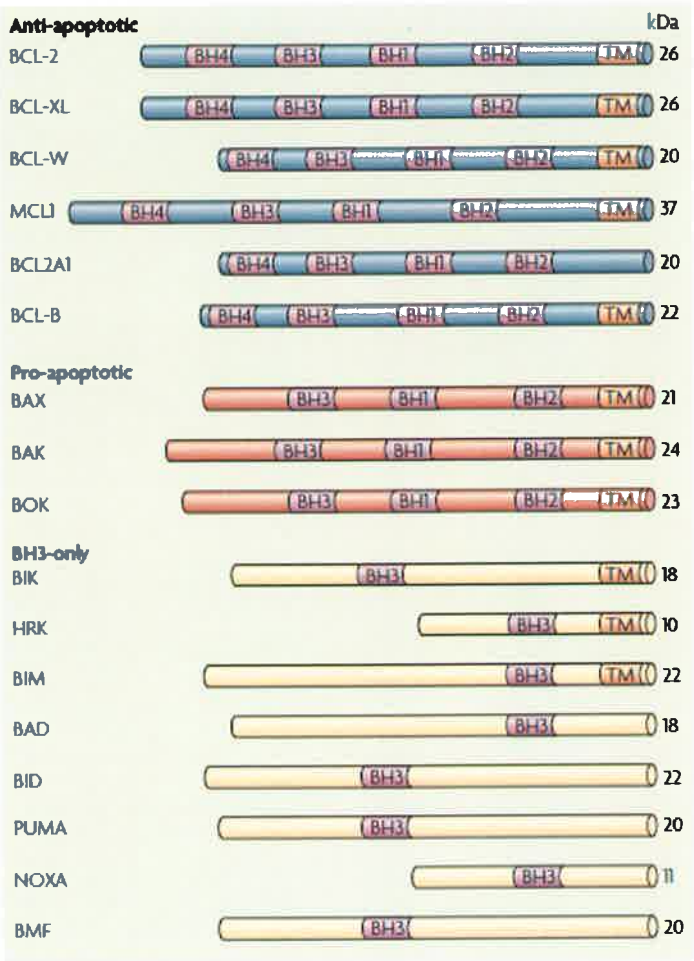


Figure 4.3: BCL-2 Family proteins; **Figure taken from Nature Reviews molecular cell biology, Vol 9, March 2008, 233**

The pro-apoptotic BAX-like sub-family share homology at BH1, BH2, BH3 but lack BH4 domains and promote apoptosis by forming pores in mitochondrial outer membranes. When these members are activated they change from monomers to oligomers and these then disrupt the integrity of the outer mitochondrial membrane, a process called mitochondrial outer membrane permeabilization

MOMP, releasing components such as cytochrome c, and ultimately initiating apoptosis[163]. The BH3-only subfamily is a structurally diverse group of proteins that only display homology within the small BH3 motif.

4.4.1 BH3-only proteins and pathway

The mammalian BH3-only protein family currently consists of up to eight members which include BID, BAD, BIM, BIK, BMF, NOXA, PUMA and HRK. All of these members are able to promote apoptosis when they are over expressed[168]. These proteins share little sequence homology apart from the BH3 motif, and are regulated in distinct ways. Heterodimer complex formation occurs through binding of the pro-apoptotic proteins's BH3 domain into the hydrophobic cleft of anti-apoptotic proteins. The BH3 mimetics are small molecule antagonists of the anti-apoptotic BCL-2 members that function as competitive inhibitors by binding to their hydrophobic cleft (Figure 4.4). Under certain conditions, antagonism of anti-apoptotic BCL-2 family proteins can unleash pro-death molecules in cancer cells. Thus, The BH3 domain mimetics are a new class of cancer drugs that specifically target a mechanism of cancer cell survival to selectively kill cancer cells[163]. Alternatively, apoptosis of cancer cells can be forced by the delivery of one member of the BH3-only protein family, or its BH3 domain alone.

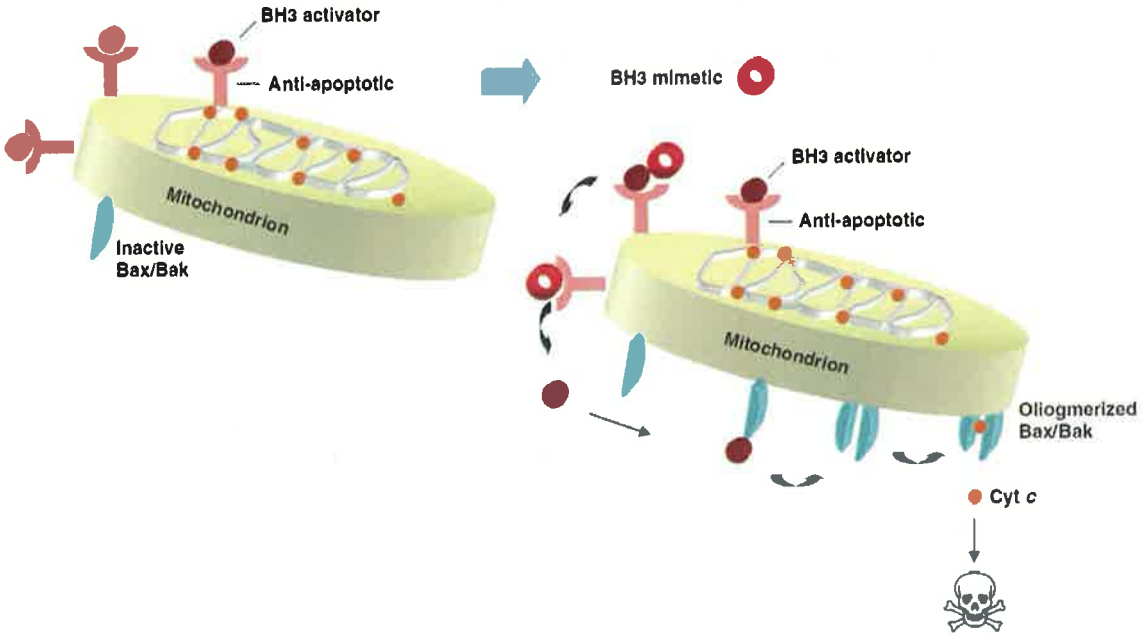


Figure 4.4: BH3 activation to release cyctochrome c and promote cell death; Figure taken from Oncogene, 2009, 27, S149-157

4.5 Cell Penetrating Peptides

Over the past decade, in order to circumvent limitations of small drug molecule- and gene- based therapies, there has been a dramatic increase of large therapeutic molecules which do not follow Lipinski's rule. These therapeutics include proteins, peptides and nucleic acids but there are restrictions in their development which include poor stability *in vivo*, lack of cellular uptake and insufficient capability to reach targets[165]. These issues maybe associated with the complete loss of pharmaceutical potency or at least with the requirements for high doses and risk of major side effects. As quoted by Heitz and co-workers in the British Journal of Pharmacology, drug delivery is still a major piece of the therapeutic puzzle still to be worked out and has an increasing demand for more efficient drug delivery systems. The major rules in drug delivery that have to be satisfied are, efficient delivery in diverse cell lines, rapid endosomal release, ability to target, low dose activity, lack of toxicity and the facility of therapeutic applications[165]. Substantial progress has been made in the design of new drug technologies to improve the uptake of

therapeutic compounds. Among these a number of non-viral strategies have been proposed, which include lipid, polycationic, nanoparticle and peptide based technologies. However, only subsets of these have been efficiently applied *in vivo* at clinical or pre-clinical levels. Of these, protein transduction domains (PTDs) or cell-penetrating peptides (CPPs), have shown to be the most promising strategy to overcome both extracellular and intracellular limitations of various biomolecules including plasmid DNA, oligonucleotides, siRNA, peptide nucleic acid (PNA), proteins, peptides as well as liposomes[172]. CPPs can trigger the movement of a biological cargo across the cell membrane into the cytoplasm of cells and improve its intracellular routing thereby facilitating interactions with the target.

4.5.1 History of Cell Penetrating Peptides

In 1988, the first PTD to be observed was by Frankel and Pabo, who showed that the transcription transactivating (Tat) protein of HIV-1 could enter cells and translocate into the nucleus[173]. Following on from this work the Prochinantz group demonstrated that Drosophila Antennapedia homeodomain could be internalised by neuronal cells, which in turn resulted in the discovery of the first PTD or CPP, a 16mer peptide derived from antennapedia termed penetratin. In 1997, work by Vives and collaborators defined a minimal peptide sequence of Tat required for cellular uptake as YGRKKRRQRRR[174]. At the same time the first non-covalent CPP for delivery of nucleic acids MPG was designed by the group of Heitz and Divita and followed shortly after by the development of PEP-1 for the cellular delivery of proteins and peptides by Morris and collaborators in 2001[175]. The groups of Wender and Futaki demonstrated that polyarginine sequences were able to drive cargo into cells and proposed that their uptake mechanisms involve bidentate hydrogen bonding interactions between guanidinium groups of the arginine residues and phosphate groups in the membrane[176]. A major breakthrough in the field of CPP's came from the first proofs of concept of their *in vivo* application for the developments in the delivery of small peptides and large proteins and also for delivery of PNAs using the chimeric peptide Transportan[177]. Ever since this discovery there has a been a large number of CPPs designed to trigger the

movement of cargo across the cell membrane into the cytoplasm[178]. CPPs are generally peptides of less than 30 amino acids which are derived from natural or unnatural proteins. They can be subdivided into two main classes, those requiring chemical linkage with the cargo and those involving formation of stable, non covalent complexes. CPPs can be distinguished by their structural properties and classified as polycationic, essentially containing clusters of polyarginine in their sequence, or amphipathic[179]. CPPs are mainly derived from natural occurring sequences but they have also been synthetically made using unnatural and modified amino acids and peptide mimetics in order to help improve their stability and/or efficiently as carriers.

4.5.2 Peptide Carriers

Like CPPs there are some AMPs that are able to translocate into the cell without disruption of the cell membrane. They have been successfully used for drug delivery into mammalian cells. However, it must be noted that there is no consensus about the mechanism of cellular uptake. There is evidence to support both endocytosis and non-endocytosis pathways[180]. The observation that some AMPs can enter host cells without damaging their cytoplasmic membrane, as well as kill pathogenic agents, has also attracted attention for use as both anticancer agent and a vector[181]. The capacity to translocate across the cell membrane has been reported for some of these AMPs. Similarities between CPPs and AMPs prompted a debate if the two classes of peptides really belong to unrelated families.

4.5.3 Buforin IIb

Buforin II is a 21 amino acid residue α-helical AMP, with complete sequence identity to the N-terminus region of histone H2A. Buforin IIb was derived from Buforin II and is a synthetic analog of the buforin II parent compound[164]. It contains a proline hinge between the two α-helices and a α-helical sequence at the C-terminus $(rllr)_3$ and displays an improved antimicrobial activity over its parent peptide. Buforin II and Buforin IIb employ a unique mechanism of activity that does not involve

membrane disruption and/or damage. This is unlike most AMPs which kill bacterial or cancerous cells by interfering with their cytoplasmic membranes and eventually causing the cell to lyse[167]. This makes buforin the perfect candidate to act as a vector for the BH3 domain while also independently having its own cyctotoxic activity. Buforin IIb acts indeed on intracellular targets and kills target cells by inhibition of transcription or translation through interaction with nucleic acids. As with any therapeutic candidate, a central issue is whether resistance can be provoked. The fact that buforin IIb has targets within the cell, as well as its fundamental interaction with the membrane, means the chances of resistance by target modification are slim, as this would require the complete alteration of the membrane or bypassing of several biochemical pathways.

4.6 Chemical synthesis of a heterodimer peptide

Two different strategies were used to develop the heterodimer peptide in a significant yield, to ultimately proceed for biological testing. The first approach was to synthesise the individual, modified, peptides and perform their purification prior to their conjugation in solution via disulphide bond formation using their cysteine residues. This process required an additional purification after combining the two peptides. The second approach was developed to address the low yielding capacity of the first approach and involved the preparation of the heterodimer by joining the two peptide sequences directly isolated from synthesis. After the crude heterodimer formation, a single purification step of the final heterodimeric peptide can be formed. The results of these two approaches are presented here.

4.6.1 Synthesis of a heterodimer peptide in a two step purification method.

The BH3 domain sequence was modified at its N-terminus with a short β-alanine linker and a cysteine residue. The entire sequence was assembled by Fmoc/*t*Bu solid phase peptide synthesis from a Rink amide MBHA resin. The cysteine residue was capped by an acetyl group on the N-terminus to prevent the presence of a

nucleophilic group on the N-terminal cysteine, yielding the final sequence Ac-C-βA-EDIIRNIARHLAQVGDSMDR-NH_2. The entire modified peptide sequence of the BH3 domain was synthesised from natural L-amino acids to ensure its interaction with the anti-apoptotic binding partner in the apoptotic pathway[163]. The β-alanine spacer was added to reduce the possibility of any steric hindrance for the conjugation of the Buforin IIB peptide via the cysteine residue. A short, 2-carbon, spacer was selected, to limit the increase in hydrophobicity of the overall peptide. Preliminary synthetic attempts, highlighted the requirements to perform a double coupling chemistry for the BH3 domain sequence after the isoleucine at position 14 in the sequence of the parent peptide, as illustrated in Figure 4.5. The modified BH3 domain and Buforin sequences written in red indicate the residues introduced by a double coupling procedure. This is due to the increased hydrophobicity in the peptide sequence and interchain association on the solid support, and double coupling was performed to ensure the highest coupling efficiency and decrease the amount of deletion peptide formed.

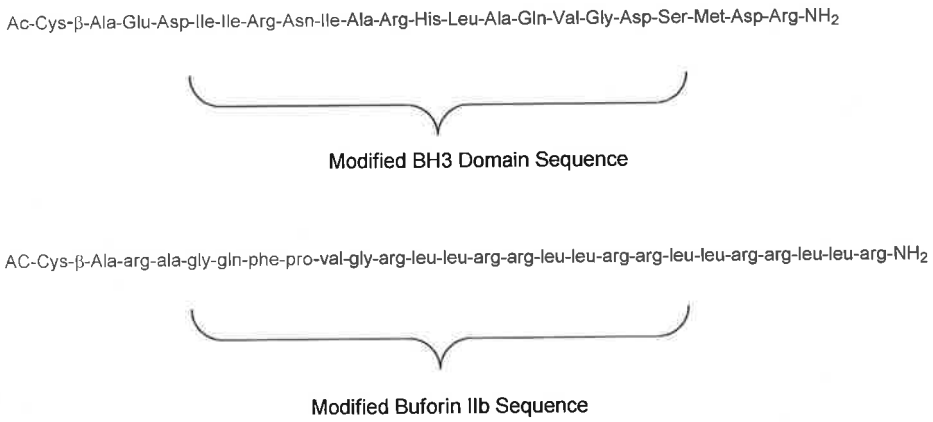


Figure 4.5: Modified BH3 domain and Buforin sequences with red colour indicating residues added by a double coupling procedure

The Buforin IIB sequence, also synthesised by Fmoc/*t*Bu strategy from a Rink amide MBHA resin, was synthesised from unnatural D-amino acids and elongated with a β-alanine linker and the N-terminal cysteine. The latter was introduced as a L-amino

acid. The N-terminus of the sequence was also acetylated giving the sequence of Ac-C-βA-raglqfpvgrllrrllrrllr-NH_2. Double coupling to assemble the sequence was performed from the proline residue until the end of the sequence for the same reasons as for the BH3 sequence, as indicated on Figure 4.5. The cleavage conditions of the peptide required air sensitive controls to limit dimerisation of the sequences through disulfide bond formation, despite the low pH conditions, and was performed under an argon atmosphere. The cleavage cocktail consisted of TFA 85%, EDT 2.5%, TA%, 2.5%, TIS 5.0% and 5% H_2O and was left stirring for 2.5hours for the BH3 peptide and up to 4.5hours for the buforin peptide due to the high number of 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) protecting groups on the side-chain of the arginine amino acids.

The two peptides where purified by semi-preparative RP-HPLC on a C18 column and analysed by mass spectrometry. Their purity was analysed by analytical RP-HPLC on a C18 column (Table 4.1).

Table 4.1: Modified BH3 and Buforin with Mass, Yeild and HPLC retention time

Compound	Exact Mass (g/mol)	Mass Found (g/mol)	Crude (mgs)	Purified (mgs)	Retention Time (min/sec)
AC-C-βAal-BH3-NH_2	2524.869	2525.0650	101	27	27.87
AC-C-βAal-Buforin-NH_2	2776.448	2776.4080	82	20	29.10

In order to favour the formation of the heterodimer peptide over the homodimer peptides, DTNP was used to activate one of the peptide's sulfhydryl groups[166]. BH3 was chosen for activation as BH3 was purified in a higher yield. This would allow for the maximum formation of the heterodimer peptide.

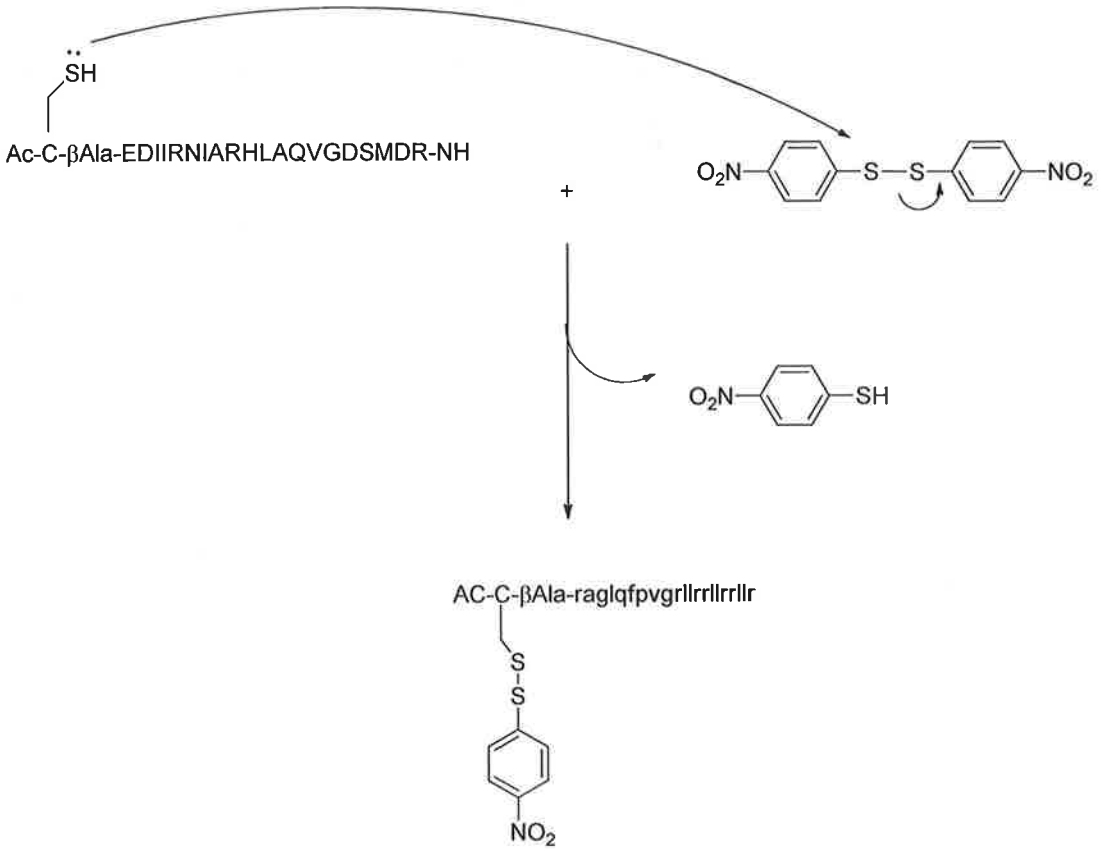


Figure 4.6: Activation of Ac-C-β-Ala-BH3-NH_2 by DTNP forming Ac-C(pNpys)-β-Ala-BH3-NH_2

DTNP acts as an activating reagent for the thiol function of the cysteine in order to synthesise heterodimeric peptides. The activating group 5-nitro-2-pyridinesulfenyl or pNpys is generated. The *para* nitro group increases the acidity of the corresponding thiol, making it a good leaving group and therefore able to react at a lower pH. The pNpys group was introduced with initially 5 equivalents of DTNP for 6 hours as described by Rabanal and collaborators. The reaction was monitored by RP-HPLC and indicated that insufficient equivalents of reagents were used to allow the reaction to go to completion in a timely manner. Figure 4.7 shows the RP-HPLC spectra of BH3 Peptide alone (a) and BH3 reacted with DTNP after 6 hours with a significant amount of peptide unconjugated (b).

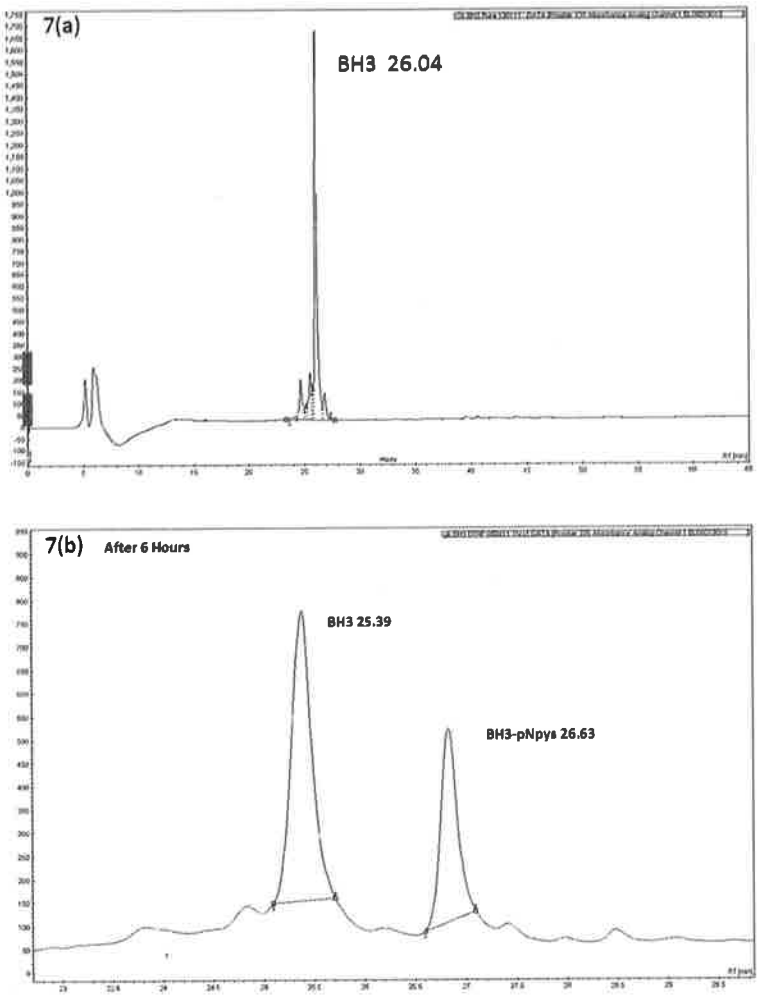


Figure 4.7: RP-HPLC spectrum of BH3 (a) and BH3 activated with DTNP (b) over 6 hours.

Optimisation of the reaction showed that up to 20 equivalents of DTNP were required for the reaction to go to completeness over a 24 hour period, as shown in Figure 4.8. After work up, the integrity of the product Ac-C(pNpys)-β-Ala-BH3-NH_2 was confirmed by mass spectrometry.

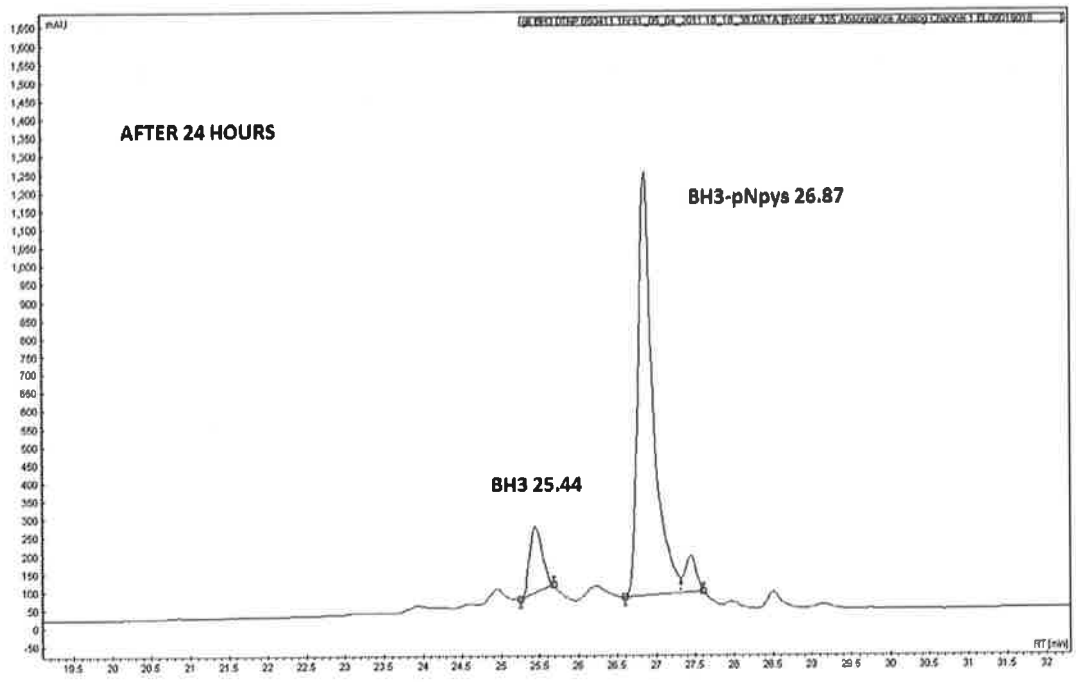


Figure 4.8: Optimised RP-HPLC of BH3 activation with DTNP

The activated BH3 peptide AC-C(pNPys)-β-Ala-BH3-NH_2 can selectively be conjugated with the Ac-C-βAla-Buforin-NH_2 to form the heterodimer, releasing in this heterodimeric disulfide bond formation the pNpys group in a S_N2 type reaction, as schematised in Figure 4.9.

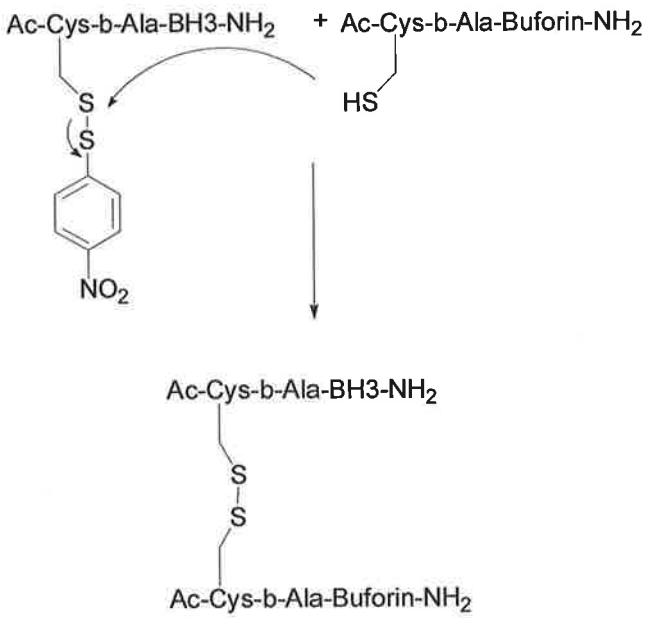


Figure 4.9: Formation of Heterodimer peptide

The heterodimer peptide was successfully produced, Figure 4.10, but its formation was accompanied by a significant amount of undesired side-products and required the purification of this final product.

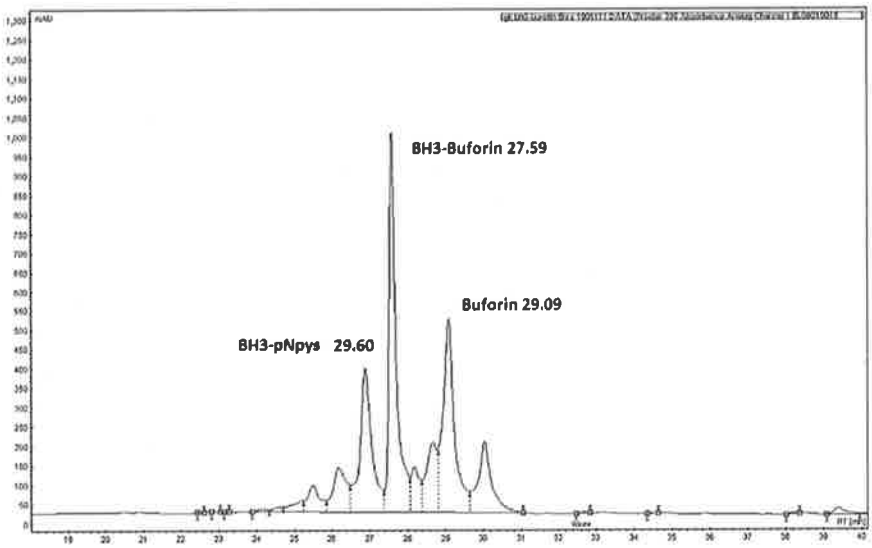


Figure 4.10: Crude BH3-Buforin peptide formation

This was performed by RP-HPLC and mass spectra analysis was obtained to confirm the integrity of the desired heterodimer peptide, Figure 4.11. However, the yield was extremely low (2.7mgs) and together with an insufficient purity 59.8% (Figure 4.11), despite purification, did not substantiate biological testing. An alternative purification method was adopted to maximise the yield and purity of the heterodimer peptide.

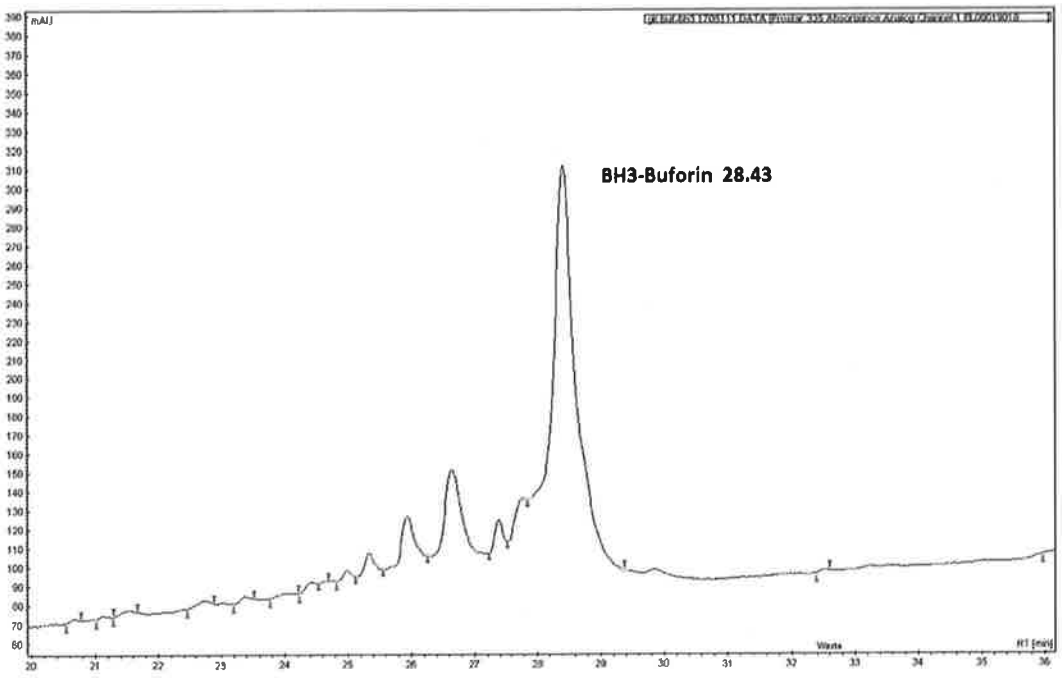


Figure 4.11: RP-HPLC spectrum of purified BH3-Buforin

4.6.2 Synthesis of heterodimer peptide in a one step purification method

After reviewing the crude peptides and the optimising steps involved in the synthesis of the individual peptides, a new approach of synthesising the heterodimer with a one step purification process was investigated. The purities of the individual peptides were sufficient to proceed to the cross-linking step without prior purification and with the activation step being also optimised, it was expected that the final purification step would allow the isolation of the desired product in purities and quantities eligible for biological testing. It was also decided to change the linker from β-alanine to hexanoic acid (Ahx), a 6 carbon spacer expected to decrease any steric hindrance involved in the formation of the heterodimer. The reaction steps were monitored by RP-HPLC and performed by omitting the intermediate purification step of the individual peptides. Also the modified buforin peptide was used for DTNP activation, as its crude peptide was obtained in higher yield and purity than the BH3 peptide (Figure 4.12(a)).

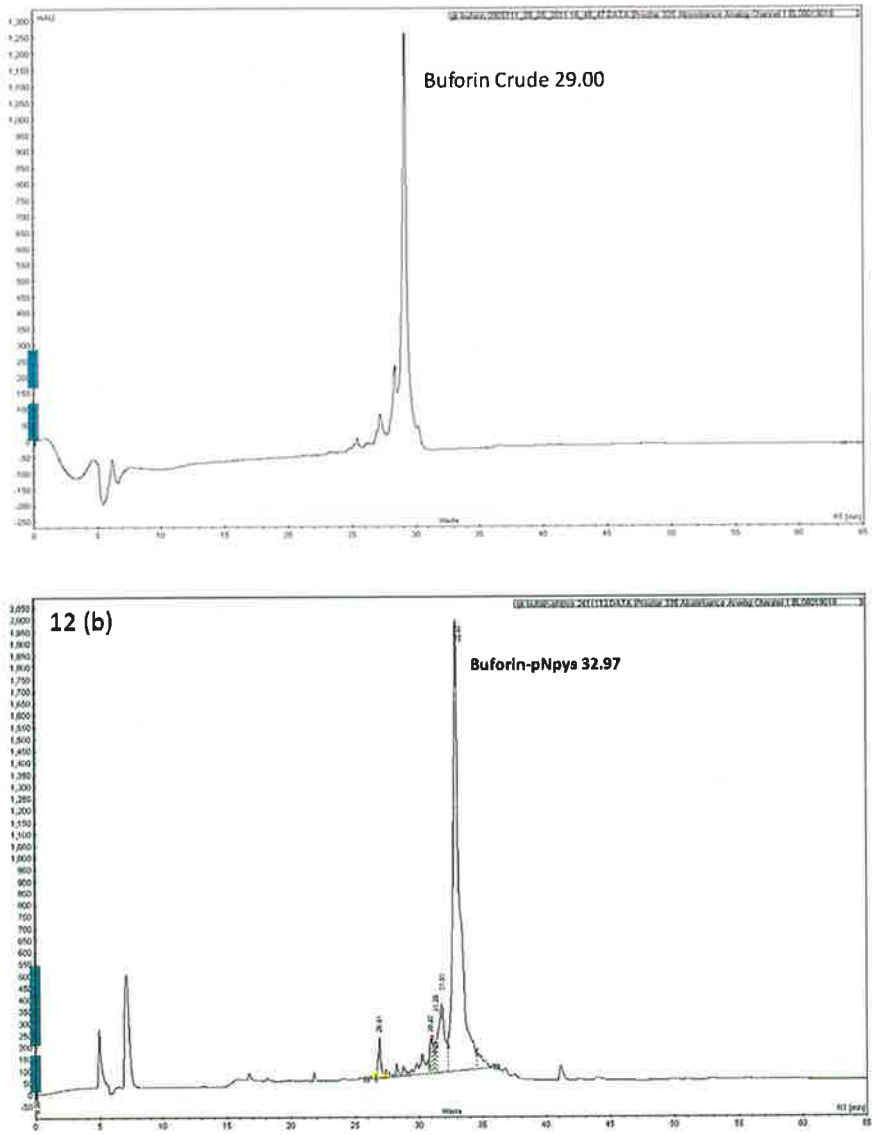


Figure 4.12: RP-HPLC spectrum of Buforin (a) and Buforin-pNpys (b)

pNpys-activated buforin (Figure 4.12(b)) was then reacted with the crude BH3 peptide according to the conditions developed in the two step purification method. The RP-HPLC of the crude and RP-HPLC purified heterodimer are shown in Figure 4.13.

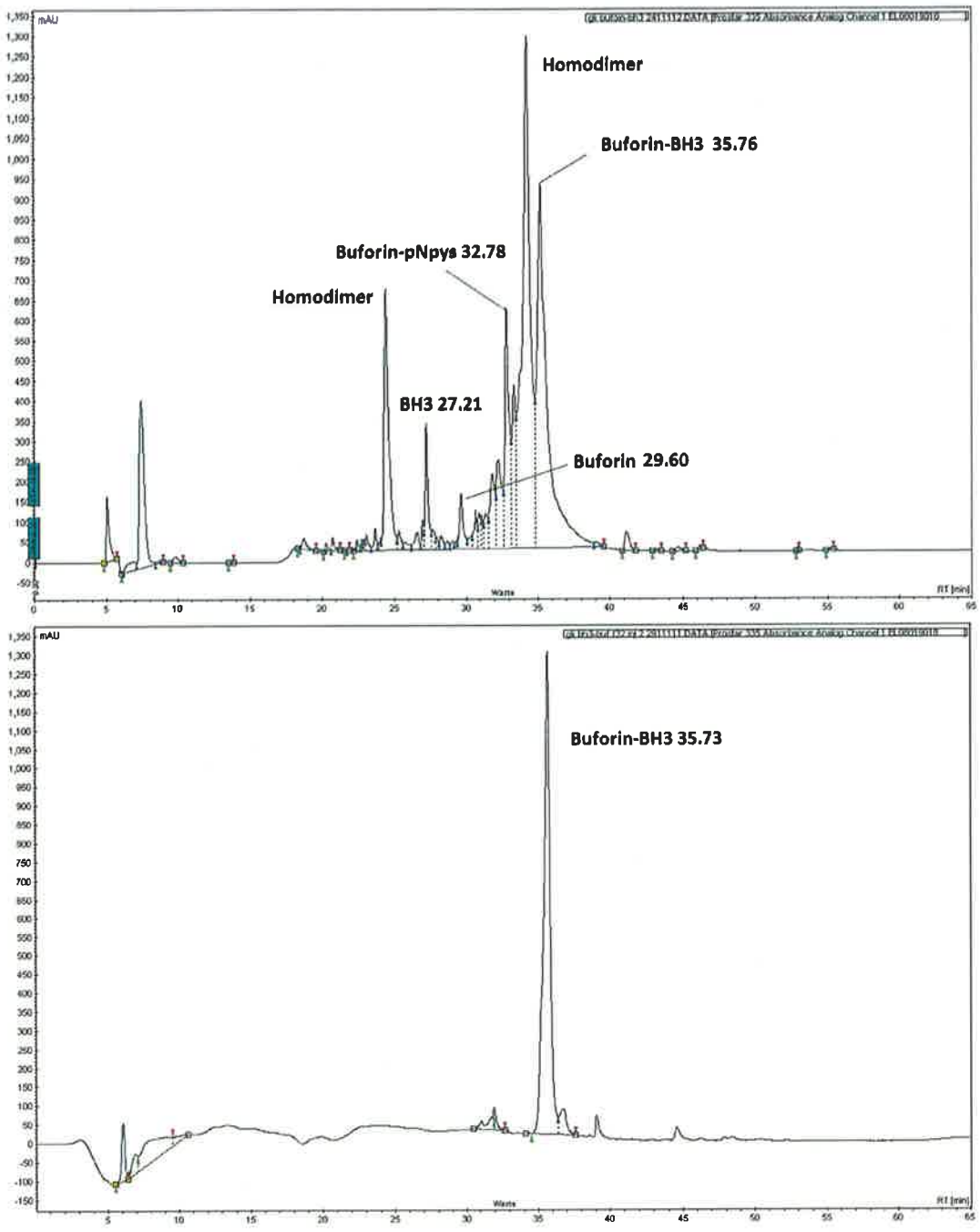


Figure 4.13: RP-HPLC spectrum of crude Buforin-BH3 and Pure BH3-Buforin

The pure Buforin-BH3 heterodimer was characterised by MALDI-TOF mass spectrometry, as shown in Figure 4.15.

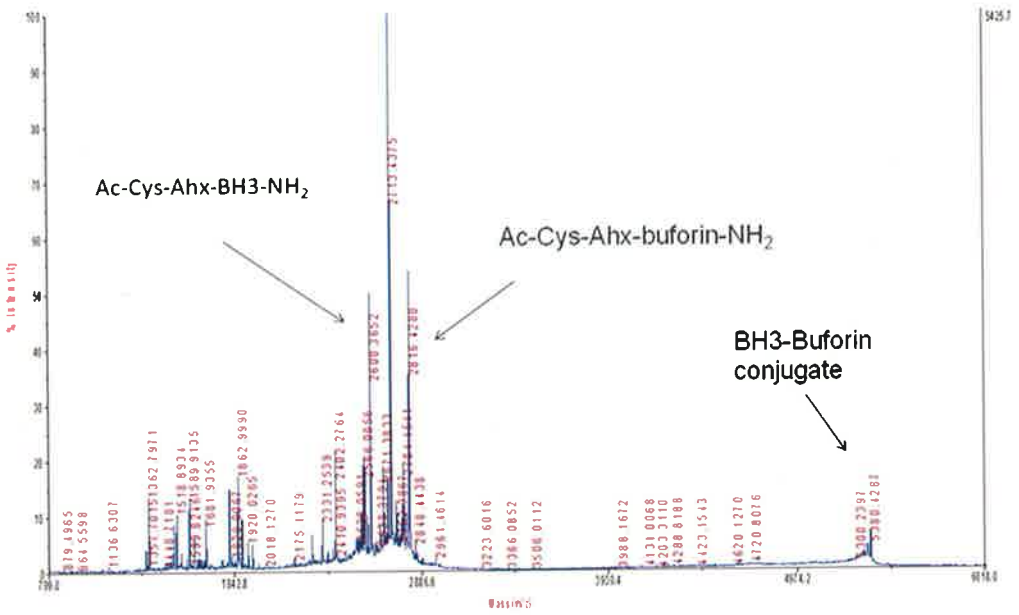


Figure 4.14: MALDI TOF BH3-Buforin conjugate with Ahx linker

The heterodimer was obtained in higher yield (27.6mgs) and purity (97.6%) from this single final purification approach and the amount isolated could sustain the biological testing of this heterodimer as an anticancer candidate.

4.7 Preliminary Biological testing and conclusion

The focus of the biological testing of this heterodimer peptide would be to demonstrate that the buforin sequence can mediate the delivery of the BH3 domain of Bid to cancer cells, while enhancing the activity of the latter, therefore that buforin can have a dual vector-anticancer agent capacity. Studies previously performed with the Bid BH3 domain were using a fatty acid modification or a fusion peptide of a CPP and BH3, which can have impact on the anticancer activity of the BH3 peptide alone and which can be exposed to resistance issues. Furthermore, these fatty acid or CPP modifications remain permanently associated with the BH3 domain. Preliminary testing was conducted in collaboration with Prof. Jochen Prehn in the Department of Physiology, Royal college of Surgeons in Ireland. The cysteine-modified buforin peptide alone was used a control peptide. Cytotoxicity assays

were set up to monitor cell death against HCT116 and HCT 116 $BAX^{-/-}$ $BAK^{-/-}$ (human colon carcinoma) cell lines. The HCT 116 $BAX^{-/-}$ $BAK^{-/-}$ cell line were cultured without BAX or BAK pro-apoptotic proteins to elucidate the contribution of the BH3 domain in the apoptosis process. Preliminary results suggest that the BH3 is indeed being carried into the cell by buforin according to a cellular uptake assay although further cell imaging will be needed to confirm the uptake. In addition, the heterodimer was shown to induce increased cancer cell death, compared to the buforin peptide alone, indicating thereby that this peptide can not only deliver a BH3 pro-apototic domain, but also enhance its anticancer activity through its own cytotoxic activity. This would require tagging of the peptide with a fluorescent dye and using confocal microscopy. The preliminary results with the HCT 116 $BAX^{-/-}$ $BAK^{-/-}$ cell lines also suggest that the BH3 apoptotic pathway is being activated by the BH3 domain sequence, with the cytotoxicity levels indicating this pathway. The Buforin peptide alone was used as a control and the Buforin-BH3 peptide showed higher toxicity against the HCT 116 $BAX^{-/-}$ $BAK^{-/-}$ cell lines. This is surprising as the proteins to activate the apoptosis have been knocked down and so therefore you would expect lower activity (Figure 4.15).

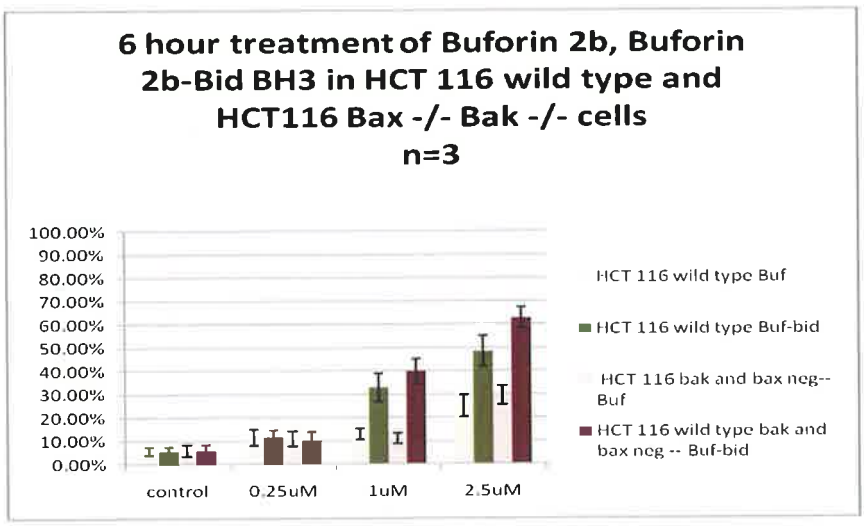


Figure 4.15: 6 Hour treatment of Buforin 2b, Buforin-BID BH3 in HCT 116 wild type and HTC116 Bax $^{-/-}$ Bak $^{-/-}$ cells.

Due to the complexity of the apoptosis pathway it is thought that the BH3 domain of the heterodimer might even interfere with a different pathway. Future work is

needed to be carried out on the project to confirm the initial hypothesis, however early indications are promising.

Chapter 5

Experimental Chapter

5 Experimental Chapter

5.1 General Procedures

5.1.1 HPLC analysis

Chromatographic analysis was performed on a perspective Biosystems Biocad SPRINT or a Varian HPLC Galaxy workstation using a variety of different reverse phase and size exclusion chromatography columns. Columns used for Reverse phase chromatography were; C-18 Gemini and C5 Jupiter columns, Phenomenex (250mm x 2.5 mm). Purfirication and analysis was ran at a flow rate of 1 mL/min or with a linear gradient over 30mins. UV detection was at 214nm unless otherwise stated and a dual wave wavelength at 280nm was used in order to detect conjugation on side chains of the peptides. Solvent A consisted of water containing, 0.1% TFA and solvent B, acetonitrile containing 0.1% TFA.

For size exclusion chromatography, BioSEP s2000 (300mm x 6.5 mm) from Phenomenex with an isocratic gradient was used with a flow rate of 1mL/min for 45 minutes with a PBS buffer 0.05molar, pH 2-8 with 50% acetonitrile to reduce the back pressure. Water was milli-Q quality and was obtained after filtration through a nylon membrane filter (0.5 μm pore).

5.1.2 HPLC purification

RP-HPLC purifications were performed on a Perseptive Biosystems Biocad SPRINT with UV detector set for dual absorbance using a semi-preparative column Gemini phenomenex C18 reversed phase chromatogram (250mm x 10mm) at a flow rate of 4 mL/min with a linear gradient programmed for a 30min run time from 5% to 65% of solvent B. Solvents were degassed by Helium bubbling for 20 min. UV absorbance was monitored at 214nm and 280nm unless otherwise stated.

5.1.3 Mass spectrometry analysis

Mass spectrometry was performed by Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) on a Reflex Bruker Spectrometer. Two matrix styles were used, DHB and hydroxyl cinnamic acid, dissolved in 50% acetonitrile and 50% water at a concentration of 10mg/mL and 15mg/mL, respectively. 1 μL of each 1:1 solution of matrix peptide were applied on the plate.

Electron Spray ionization (ESI-MS) mass spectrometry were obtained on a Walters Micro mass LCT and low resolution mass spectrometer were recorded on either Walters Micro mass Quattro LCMS spectrometers at 80 eV.

5.1.4 NMR spectrometry (NMR)

NMR spectrometry was recorded on a Bruker Avance 400MHz spectrometer. Samples were prepared in $CDCl_3$ (referenced to 7.26ppm for ^{1}H and 77.0 for ^{13}C), DMSO-d6 (referenced to 2.51ppm and 3.3 ppm for ^{1}H and 40 for ^{13}C), MeOD (referenced to 3.31 ppm for ^{1}H and 49.0 for ^{13}C) and D_2O (referenced to 4.79 ppm for ^{1}H). Coupling constants (J) are in Hertz and corrected to the nearest 0.5 Hz. Multiplicities are reported as follows: s, singlet, d, doublet, dd, doublets of doublets, t, triplet, q, quartet, m multiplet, c complex, br broad. ^{1}H NMR spectra are supported by DEPT analysis where necessary.

Reactions were checked for completion by TLC (EM Science, silica 60 F 254) which were visualised by quenching of U.V. fluorescence (λ_{max}= 254 nm). Flash Chromatography was performed using *silica gel* 60 (0.040-0.063 mm, 230-400 mesh) or by alumina oxide, activated, basic Brockmann 1, standard grade ~ 150 mesh 50 Å. Iodine crystals were also used for TLC staining in the presence of PEG compounds.

5.1.5 Infrared Spectrometry (IR)

Infrared spectra (IR) were recorded from KBr discs using a Bruker Tensor27 FT-IR instrument. Absorption maximum (ν_{max}) was recorded in wave numbers (cm^{-1}) and only selected peaks are reported.

5.1.6 Ultraviolet Spectrometry (UV)

Ultraviolet Spectrometry (UV) where recorded in quartz cuvettes of 1cm length using a biochrom Libra S22 instrument. De-ionized water was used as a blank unless otherwise stated.

5.1.7 General procedure for Peptide synthesis

The Assembly of the peptides were performed either by manual or automated Solid Phase Peptide Synthesis (SPPS) using standard fluorenylmethoxycarbonyl/*tert*-butyl (Fmoc/*t*Bu) protection strategy. The automated synthesis was performed on an Applied Biosystem ABI 433A Synthesizer.

Manual Synthesis were performed was as follows: Resin was placed in a fritted reaction vessel and the lid closed. The maximum scale, depending on the resin type and the swelling properties of the resin was 0.1-0.25 mmol. The resin was swelled in DCM beforehand to ensure maximum swelling and agitated for 20minutes. The DCM was drained and then the resin was swelled in DMF for 10minutes. The Rink Amide MBHA (methylbenzhydrylamine linker) resin, the NOVA PEG resin, Sieber resin and Rink Amide PEGA resin was used for C-terminus amidation of peptides.

Deprotection of Fmoc protected amino acids was performed in 20-25% of piperidine in DMF. Approximately 10mls of the deprotection solution was added and placed on the shaker for 10mins, it was then drained and 2 more treatments with fresh solution for 5 minutes were preformed. The resin was then washed 3 x 5min with DMF and 1 x 5min with DCM. Deprotection was monitored by Kaiser test.

5.1.7.1 Coupling of Fmoc protected amino acids

Coupling of the Fmoc protected amide groups (amino acids/PEGs) were performed on solid support once the free amino was exposed after deprotection or if a free amine was already present. The equivalents of amino acid to coupling reagents used were based on the substitution of the resin. DIPEA was always double the amount of equivalents of Fmoc protected amino acid/PEG. The coupling reagents equivalents were always slightly lower than the equivalents of the Fmoc-amino acid/PEG conjugates (e.g. 2 eq. amino acids/PEG 1.9 eq of coupling reagent). Different coupling reagents were used depending on which amino acid /PEG was being attached. Coupling Reagents included HOBt/HBTU, HATU, Pybop/HOBt. Coupling reagents and amino acids/PEG were dissolved in DMF/NMP and brought up to approx 10mls and placed on the shaker for 1-3hours. Coupling was also monitored by Kaiser test and the elimination of free amine was observed.

5.1.7.2 Cleavage of peptide and PEG-peptide from resin

Following chain assembly the resin was dried by washing in DCM and then left to air dry. The resin was then transferred to 25ml round bottom flask and a cocktail cleavage solution was prepared. It's important to make the solution up fresh and in a separated vessel before adding to the resin. The general cleavage cocktail included trifluoroacetic acid (TFA) 85%, 5% triisopropylsilane (TIPS), 2.5% thioanisole (TA), 2.5% ethanedithiol (EDT) and 5% water. The cleavage cocktail and reaction time of the cleavage was adjusted to accommodate the side chain deprotection of different amino acids with particular attention given to sulphur containing amino acids and also to arginine. The minimum amount of time for the cleavage was 2 hours and an additional 30minutes for every arginine in the sequence. After filtering the solution from the resin the peptide/PEG-peptide was precipitated in a minimum amount of cold diethyl ether and frozen for up to 10 minutes followed by centrifugation for 5mins at 2.8×10^3 rpm. The liquid phase was precipitated washed and centrifuged again with cold diethyl ether twice more. The

peptide was left to air dry, dissolved in water, frozen in liquid nitrogen and placed a freeze dryer. The lyophilized powder is then ready for purification. If the peptide is too hydrophobic and won't precipitate, the peptide was then placed under N_2 and the cleavage cocktail and diethyl ether were evaporated off leaving the peptide and trace amount of scavengers in the flask for freeze drying.

5.1.8 General procedure for cell culture

5.1.8.1 Cell culture

Mouse mammary adenocarcinoma (breast cancer) cells (4T1.2 Luc) and lung carcinoma (A2780p) cell lines were kindly provided by Dr Judy Harmey and Dr Darren Griffith, respectively (Royal College of Surgeons in Ireland). 4T1.2 Luc cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/L glucose, supplemented with 10% (v/v) heat inactivated Foetal Bovine Serum (FBS), (BioSera, UK) whilst the A2780p cell line was maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 100 µg/ml streptomycin, 100 U/ml penicillin and 10% (v/v) FBS (all from BioSera, UK). Hereafter, this will be referred to as complete RPMI 1640 medium. All cells were maintained at 37°C in a 5% CO_2 humidified atmosphere.

Cells used in collaboration with IT Tallaght (Dr Folytyn-Arfa Kiab and Dr Siobhan McClean) were two ovarian cell lines, one malignant (SK-OV-3) and one non malignant (HS-832). The SK-OV-3 and the Hs832 cells lines were both grown in Dulbecco's Modified Eagle's Medium (DMEM) with 2mM glutamine, supplemented with 100 µg/ml streptomycin, 100 U/ml penicillin and 10% (v/v) heat inactivated Foetal Bovine Serum and (FBS), (BioSera, UK). All cells were maintained at 37°C in a 5% CO_2 humidified atmosphere.

Cell lines used in collaboration with RCSI ERC Beaumount (Dr Warren Thomas) where three primary lung cancer cell lines, REN (epithelioid-type mesothelioma), Msto-211H (streptomycin, 100 ug/ml penicillin and 10% (v/v) FBS (all from BioSera, UK). Hereafter, this will be referred to as complete RPMI 1640 medium. All cells

were maintained at 37°C in a 5% CO_2 humidified atmosphere, (Biphasic-type mesothelioma) and H2052 (sacomatoid-type mesothelioma) and cell lines. All cell lines were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with streptomycin, 100 ug/ml penicillin.

5.1.8.2 Cell maintenance and harvest

Cells were maintained in the appropriate media as described in the above section. Cells were routinely passaged by trypsinization in 0.5% (w/v) trypsin/0.2% (w/v) EDTA mixture in phosphate-buffered saline (PBS). To inactivate the trypsin, an equal volume of complete medium was added. Detached cells were collected by centrifugation (3 min) at 1000 rpm.

5.1.8.3 Cell counting

Harvested cells were re-suspended in an appropriate volume depending on the size of the cell pellet. An aliquot of the single cell suspension was mixed with an equal volume of 0.4 % (w/v) trypan blue and counted using a haemocytometer (Improved Neubauer model) under a phase contrast microscope. Viable cells were counted in four quadrants and the mean value multiplied by two (dilution factor) and then by factor of 10^4, which accounts for the volume of the haemocytometer. Using this method, a value representative of the number of cells per ml in the original suspension was obtained.

5.1.8.4 Cryopreservation of cultured cells

Harvested cells were first re-suspended in their corresponding complete medium containing 10% (v/v) of the cryoprotective agent dimethylsulphoxide (DMSO; Sigma). Aliquots containing approximately 1 x 10^6 cells were dispensed into cryotubes (Nalgene, Rochester, NY) and frozen using a *Cryo* 1ºC Freezing container (Nalgene). The cryotubes were then stored in the vapour phase in a ThermoForma (ThermoElectron Corporation, Ohio, USA) liquid nitrogen vessel. Cryopreserved cells

were revived by thawing rapidly in a water bath at 37ºC before immediate suspension in pre-warmed complete media.

5.1.8.5 Cell Viability assay

Cell viability was assessed by MTT/MTS colorimetric assay. The MTT assay is based on the reduction of the soluble yellow MTT salt (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), via the mitochondria of metabolically active cells, leading to the precipitation of purple water-insoluble formazan crystals. Relative numbers of viable cells were determined spectrophotometrically following solubilisation of the formazan crystals in DMSO. One day prior to treatment with test compounds, cells were seeded at a density of, as per guide lines of cell line, per well in 96-well plates, in a volume of 150 µL of media per well. The following day, media was aspirated from cells and replaced with media containing test compounds at concentrations ranging from 100 µM to 0.76 µM depending upon compounds being tested. The plates were placed in the incubator for 24, 48 or 72 h for combination studies. On the day of analysis, 50 µL of a fresh sterile filtered solution of MTT (5 mg/ml; Sigma-Aldrich) was added to each well of treated plates using a multi-channel pipette. The MTT solution was prepared using sterile PBS and filter sterilised using a 0.2 µm filter (Millipore). Plates were then returned to the incubator for 4 h. The media/MTT solution was aspirated from the well, with care taken not to dislodge the formazan crystals from the bottom of each well. The crystals were dissolved following addition of 200 µL DMSO (Sigma-Aldrich) to each well. Finally, absorbance of the resulting solution was measured at 570 nm for 1 second using a microplate reader (Perkin-Elmer). MTS is a similar but is a one step process. After the incubation period of 24, 48 or 72 h, 30µL of MTS was added to the wells and the plates were returned for incubation for 3hrs and the absorbance was read at 490 nm for 1 second. Absorbance values in treated plates were expressed as a percentage of untreated controls in order to obtain percentage viability values. The percentage viability values were plotted against the log concentration and a sigmoidal log dose curve was calculated by non-linear

regression analysis using Graph pad Prism software version 5.0 for Windows (GraphPad Software, CA, USA).

5.1.8.6 Procedure for purified enzyme assay

The purified Enzyme, Cathepsin B, was dissolved in PBS, EDTA 0.05M solution and 100µL was pipetted into a well of a 96 well pate. Prodrug and control peptides where dissolved in water (1mg/mL) and 100µL was pipetted onto the cathepsin B solution and placed in an incubator and maintained at 37°C in a 5% CO2 humidified atmosphere for 24 h. The reaction was monitored by RP-HPLC and Mass Spectrometry showing the cleavage and release of the peptide prodrug. The Control peptide displayed no degradation up to 48 h.

5.1.9 Materials and Equipment

Materials and reagents were purchased from commercial suppliers and used without additional purification, unless otherwise stated. Protected amino acids were purchased from Novabiochem (Merck biosciences, Hohenburnn, Germany), Iris Biotech GmbH (Marktredwitz, Germany). Coupling reagents were purchased from Novabiochem; HBTU, PyBop and HATU and from Iris Biotech GMbH; HOBt and HATU. All resins were also purchased from Novabiochem (Rink Amide MBHA, Rink Amide MBHA PEGA Resin, Sieber resin and Nova PEG resin). Solvents (NMP, DCM synthesizer grade) where purchased from Applied Biosystems (Warrington United Kingdom). PEGs were purchased from Iris Biotech GMbH and JenKem (California, USA). Doxorubicin was purchased from Merck biosciences. All other reagents and solvents, including HPLC-grade acetonitrile, were obtained from Sigma-Aldrich Ireland.

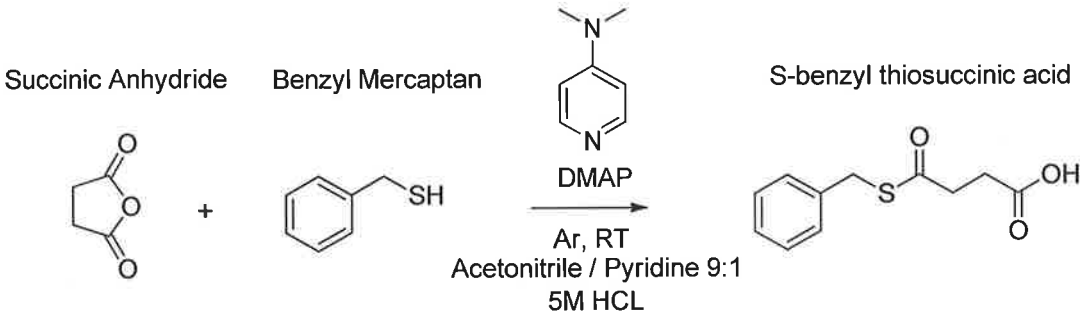
5.1.10 Statistical Analysis

All cell work was performed in triplicate and n=3. Graphpad Prism 5 was used to determine the IC_{50} values. Statiscal analysis was carried out using standard student T-test or by two-way ANOVA.

5.2 General Experimental Details

5.2.1 Experimental procedure for Chapter 2

5.2.1.1 Synthesis of *S*-benzyl thiosuccinic acid



*(**Caution**: Benzyl mercaptan is an extremely potent sulphur compound and must be kept in the fumehood during all steps and all glassware to be placed in a bleach bath afterwards for 24hours)*

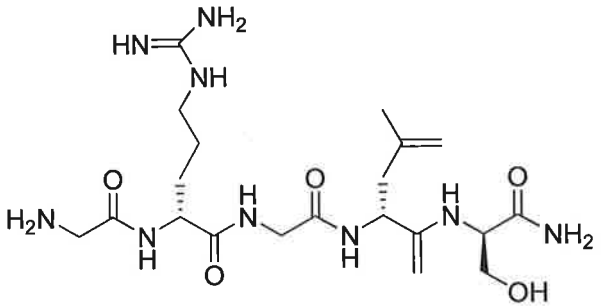
Procedure: Benzyl mercaptan (2.595µL, 22µmol) was added, under nitrogen, to a solution of succinic anhydride (2.00g, 20µmol) and 4-dimethylamino-pyridine (122.2mg, 1 µmol) (DMAP) in 25mL of anhydrous acetonitrile/ pyridine 9:1 (22.5mL/2.5mL). This was stirred at room temperature for 3 hours and was evaporated using a rotary evaporator. An oily residue was obtained. The product was dissolved in 30mL of aqueous sodium bicarbonate ($NaHCO_3$) at pH 8.5 and extracted twice with 10mL of diethyl ether. The product was cooled in an ice bath and acidified with 5N hydrochloric acid (HCL) to pH 2. This formed a white precipitate and it was filtered off using 0.1M HCl solution. The product was then

placed in a desiccator with phosphorus pentoxide to dry overnight and stored in the fridge at -4 ℃.

^{1}H NMR Bruker 400 MHz ($CDCl_3$): 2.65 (t, 2H, J=6.8 Hz), 2.82 (t, 2H, J=6.8Hz), 4.08, (S, 2H), 7.12 (m, 5H), 2.10 (s), solvent peak acetone. (Appendix 1)

Theoretical Yield: 4.48g Actual yield: 2.91g %Yield: 64.9%

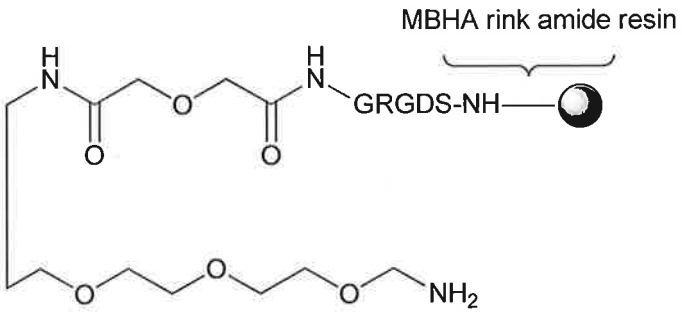
5.2.1.2 Preparation of Peptide, H-GRGD-NH_2



Procedure: The peptide with the sequence H-GRGDS-NH_2 was assembled form "D amino acids" and synthesized by Fmoc/*t*Bu strategy on MBHA rink amide resin (substitution of 0.7 mmol, 0.143g, 0.1mmol scale) by automated synthesis applied biosystems 433a. The Fmoc protected amino acids Fmoc-Gly-OH, Fmoc-D-Arg(Pbf)-OH, Fmoc-D-Asp(*t*Bu)-OH, Fmoc-D-Ser(*t*Bu)-OH were used with 10eq. An aliquot cleavage of peptide from the resin was performed to ensure the correct peptide was synthesised. The cleavage cocktail consisted of 850µL of TFA, 50µL TA, 50µL water and 50µL TIPS which was added to the resin and left stirring for 2.5 hours. The product was precipitated in diethyl ether as a white solid, collected by centrifugation and washed twice with diethyl ether. The product was dissolved in water for mass spectrometry analysis.

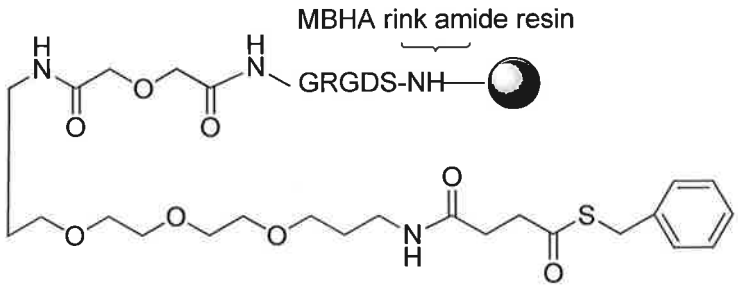
Mass Spectra results: Expected Mass= 489.48g/mol MW= ES^+ 490.3 g/mol , ES^- 488.4 g/mol (Appendix 2)

5.2.1.3 Preparation of PEGylated H-GRGDS-NH-◕



Procedure: PEGylation of peptide H-GRGDS-◕ (**compound 5.2.1.2**) was performed on the solid support by manual coupling. HOBT/HBTU/DIPEA was used to couple the Fmoc-NH-$PEG_{(20\ atom)}$-COOH to the resin on solid support. The coupling reaction was carried out using 1.9 equivalents of coupling reagents with HOBt 29.0mg (0.19mmol, 153g/mol), HBTU 72.0mg (0.19mmol, 379g/mol) and 4 equivalents of DIPEA 69.7 μL (0.76mmol, 129g/mol, Density 0.74). They were dissolved in DMF and added to 2 equivalents of Fmoc-NH-$PEG_{(20ataom)}$-COOH, 111.6mg (0.2mmol, 558g/mol). This solution was added to the peptide H-grdgs-◕ and left to agitate on a shaker for 3 hours.The coupling was monitored by Kaiser test to ensure complete coupling. Deprotection was carried out on the Fmoc protecting group using the standard deprotection protocol and again monitored by Kaiser test to ensure complete deprotection of the Fmoc group.

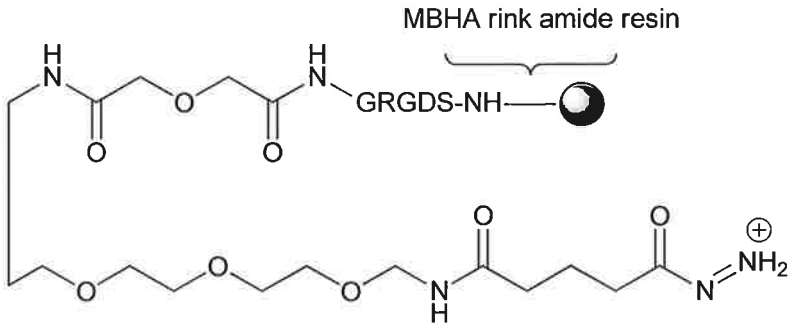
5.2.1.4 Preparation of *S*-Benzyl thiosuccinic acid conjugated to H_2N- $PEG_{(20atom)}$-H- GRGDS -NH-◕



Procedure: S-benzyl thiosuccinic acid (**Compound 5.2.1.1**) was coupled on solid support to the PEGylated peptide H_2N-$PEG_{(20ataom)}$-H- GRGDS -NH_2-◕ (0.1mmol) (**Compound 5.2.1.3**). *S*-benzyl thiosuccinic acid 112.5mg, (0.5mmol, 224g/mol) was dissolved in 2mL of DMF and added to a solution of HOBt 74.9mg (0.49mmol, 153g/mol), HBTU 185.7mg (0.49mmol, 379g/mol) and DIPEA 174 µL (1mmol, 129g/mol, Density 0.74) in DMF. Once mixed this was added to the H_2N-$PEG_{(20ataom)}$-H-GRGDS-NH_2-◕ and left to agitated on the shaker for 3 hours. The reaction was monitored by Kaiser test. An aliquot cleavage of peptide from the resin was performed to ensure correct that the peptide was synthesised. The cleavage cocktail consisted of 850µL of TFA, 50µL TA, 50µL water and 50µL TIPS, which was added to the resin and left stirring for 2.5 hours. The product was precipitated in diethyl ether as a white solid, collected by centrifugation and washed twice with diethyl ether. The product was then dissolved in water for mass spectrometry analysis.

Mass Spectra results: Exact Mass: 1014.4077g/mol GCMS electron Impact: 1014.6 g/mol (Appendix 3)

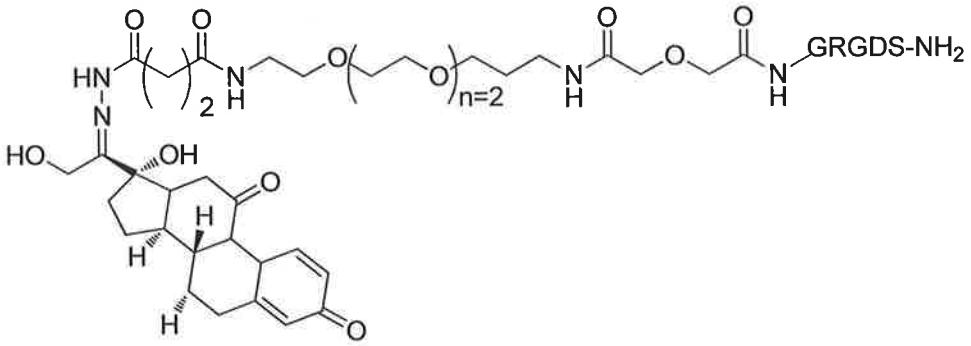
5.2.1.5 Preparation of hydrazide linker on *S*-benzyl-$C_5O_2H_4$-NH-$PEG_{(20ataom)}$-H-grdgs-NH_2-◕ for addition of ketone for click chemistry.



Procedure: 2mL of hydrazine hydrate and 2mL of 1,4 dioxane was placed in the reaction vessel with the pre swollen *S*-benzyl-succinic-NH-$PEG_{(20ataom)}$-GRGDS-◕ (**compound 5.2.1.4**) in DCM. The reaction was agitated on a shaker for 1 hour. The resin was then washed 3 x 5mins in DCM and the reaction was repeated twice to ensure the complete removal of the benzyl mercaptan protecting group. The product was then cleaved from the resin using a cocktail cleavage of 4500µL of TFA, 125µL TA, 125µL water, 250µL TIPS and 250µL H_2O and left stirring for 2.5 hours. The peptide conjugate was then precipitated in diethyl ether as a white solid and collected by centrifugation. The product washed twice with diethyl ether, dissolved in water, lyophilized overnight and a white powder was collected. A small amount of the sample was dissolved in water for mass spectrometry analysis.

Mass Spectra results: Exact Mass: 921.4152 Electron Spray: ES^+ 922.6 g/mol ES^- 920.8g/mol (Appendix 4)

5.2.1.6 The Preparation of Prednisone conjugated to H_2N=N-$C_5O_2H_4$NH-$PEG_{(20ataom)}$-H- GRGDS -NH_2 .

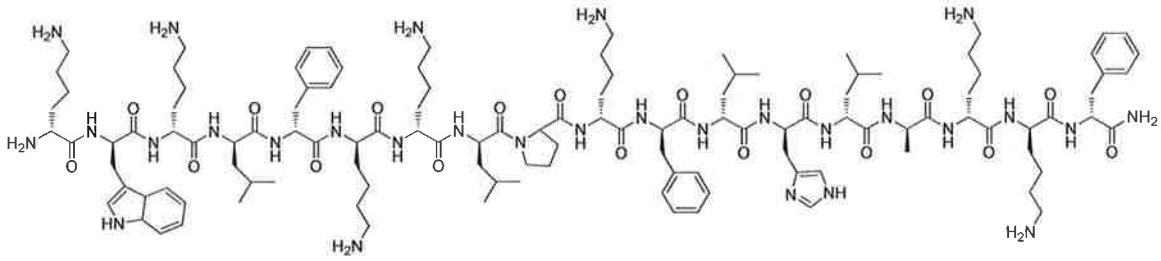


Procedure: 20mg (**Compound 5.2.1.5**) (992g/mol, 0.2mmol) was added into a round bottom flask and with prednisone 7.7 mg (358g/mol, 2.0mmol), a few drops of acetic acid and left stirring for 24hours in anhydrous methanol under argon. The methanol was then removed by rota evaporator and placed on a high vacuum line to dry completely. 1mg of the crude product was then dissolved in methanol for mass spectrometry analysis.

Mass Spectra results: Exact Mass: 1261.8432 MALDI TOF: 1262.4906 g/mol

(Appendix 5)

5.2.1.7 Preparation of peptide P18, with a sequence of H-kwklfkklpkflhlakkf-NH_2

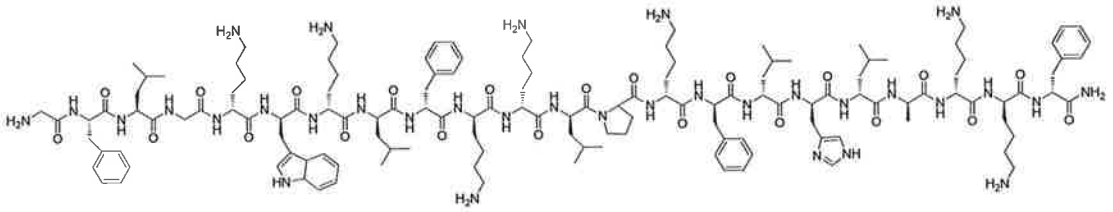


Procedure: The peptide P18 was synthesized in 'D amino acids', with the sequence H-Lys-Trp-Lys-Leu-Phe-Lys-Lys-Leu-Pro-Lys-Phe-Leu-His-Leu-Ala-Lys-Lys-Phe-NH_2 (P18) by Fmoc/*t*Bu strategy on Rink Amide MBHA resin with a substitution of 0.7mmol, 0.143g, 0.1mmol scale by automated peptide synthesis, Applied Biosystems 433a. The Fmoc protected amino acids used with 10 equivalents where Fmoc-D-Lys(Boc)-OH, Fmoc-D-Trp(Boc)-OH, Fmoc-D-Leu-OH, Fmoc-D-Phe-OH, Fmoc-D-Ala-OH, Fmoc-D-Pro-OH, Fmoc-D-His(Trt)-OH with Leu[8] being double coupled (20equivalents) after the Proline[9]. The peptide was cleaved from the resin in a cleavage cocktail solution, 5100µL TFA, 150µL EDT, 150µL TA, 300µL water and 300µL TIPS and was left stirring for 2.5 hours. The product was precipitated in diethyl ether as a white solid and collected by centrifugation and washed x2 with diethyl ether. The isolated peptide was dissolved in water and lyophilized overnight and a white powder was collected. The product was purified by RP-HPLC, on a semi-prep C18 Gemini column. Once collected, the peptide was lyophilized overnight. 1mg of the pure sample was taken for mass spectrometry MALDI-TOF analysis using α-cyano-4-hydroxycinnamic acid. The purity of the peptide was analysed by RP-HPLC, analytical C18 Gemini column.

Mass Spectra results: Exact Mass: 2300.4760 MALDI-TOF 2300.9330 g/mol

RP-HPLC Results: 98.12% pure, Retention Time: 22min 85sec (Appendix 6 + 7)

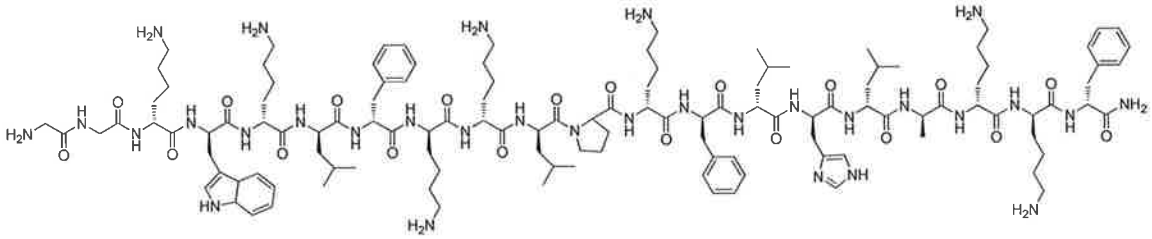
5.2.1.8 Preparation of peptide, Gly-Phe-Leu-Gly-P18. H-GFLG-kwklfkklpkflhlakkf-NH_2



Procedure: The peptide P18 was assembled in D amino acids and the protease tetrapeptide linker (Gly-Phe-Leu-Gly) with Phe^2 and Leu^3 were assembled in L amino acids. The sequence was synthesized by Fmoc/*t*Bu strategy on Rink Amide PEGA resin, substitution of 0.33mmol, 1.69g of wet resin used, 0.1mmol scale on Applied Biosystems 433a automated peptide synthesizer. The Fmoc protected amino acids were added with 10 equivalents with leu^8 being double coupled (20equivalents) after the pro^9. The tetrapeptide linker GFLG was also added on the synthesizer and double coupled (20equivalents). An aliquot cleavage of peptide from the resin was performed to ensure that the correct peptide was synthesised. A cocktail cleavage of 850µL of TFA, 25µL TA, 25µL EDT, 50µL water, 50µL TIPS was added to the resin and left stirring for 2.5 hours. The product was then precipitated in diethyl ether as a white solid, collected by centrifugation and washed x2 with diethyl ether. A small amount of the sample was dissolved in water and prepared for mass spectrometry (MALDI-TOF) analysis using α-cyano-4-hydroxycinnamic acid (dissolved in water 0.1% TFA / acetonitrile 0.1% TFA) 1:1 ratio matrix to product.

Mass Spectra results: Exact Mass: 2674.6719 g/mol MALDI-TOF: 2673.3981g/mol (Appendix 8)

5.2.1.9 Preparation of peptide control H-GG-P18, H-GG-kwklfkklpkflhlakkf-NH_2

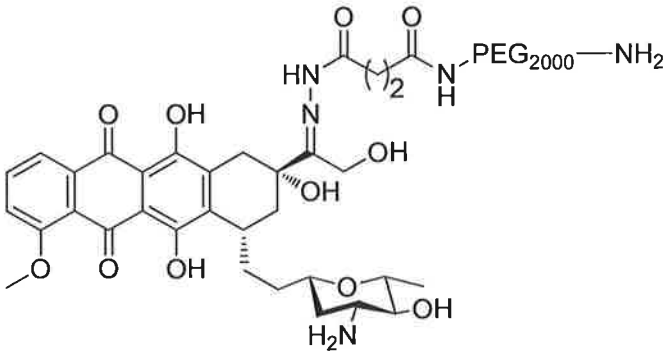


Procedure: The peptide P18 was synthesized in D amino acids by Fmoc/*t*Bu strategy on Rink Amide PEGA resin, substitution of 0.33mmol, 1.69g of wet resin (0.1mmol) on Applied Biosystems 433a automated peptide synthesizer. The Fmoc protected amino acids where using 10 equivalents with leu[8] being double coupled (20equivalents) after the pro[9]. The dipeptide linker Gly-Gly was added individually on the synthesizer (Fmoc-Gly-OH) but double coupled with 20 equivalents. An aliquot cleavage of peptide from the resin was performed to ensure the correct peptide was synthesised. A cocktail cleavage of 850µL of TFA, 25µL TA, 25µL EDT, 50µL water and 50µL TIPS was added to the resin and left stirring for 2.5 hours. The product was then precipitated in diethyl ether as a white solid, collected by centrifugation and washed twice with diethyl ether. A small amount of the sample was dissolved in water and prepared for mass spectrometry MALDI-TOF analysis using α-cyano-4-hydroxycinnamic acid (dissolved in water 0.1% TFA / acetonitrile 0.1% TFA). 1:1 ratio matrix to product.

Mass Spectra results: Exact mass: 2414.5195 g/mol MALDI-TOF 2415.8586 g/mol

(Appendix 9)

5.2.1.10 Preparation of DOX=N-NH-$C_4O_2H_4$-NH-$PEG_{(2000)}$-GFLG-P18-NH_2



Peptide H-GFLG-P18-◕ was synthesized as described in **compound 5.2.1.8.** PEGylation of peptide H-GFLG-P18-NH-◕ was performed on the solid support by manual coupling. HATU/DIPEA were used as coupling reagents for the Fmoc-NH-$PEG_{(2000)}$-COOH. The coupling reaction was performed using 1.9 equivalents of HATU (72.0mg) and 4 equivalents of DIPEA (69.6 µL) dissolved in DMF and added to 2 equivalents of Fmoc-NH-$PEG_{(2000)}$-COOH (400mg). The solution was added to the peptide H-GFLG-P18-NH-◕ and left to agitate on a shaker for 3 hours. Coupling was monitored by Kaiser test to ensure complete coupling. Deprotection was carried out on the Fmoc protecting group using the standard deprotection protocol and again monitored by Kaiser test to ensure complete deprotection of the Fmoc group.

Mass Spectra results: Before deprotection: MALDI-TOF: 5024.0430 g/mol
After deprotection: MALDI-TOF: 4801.2998 g/mol (Appendix 10,10a and 11,11a)

Addition of S-benzyl thiosuccinic Acid

S-benzyl thiosuccinic acid (**Compound 5.2.1.1**) 112.5mg (0.5mmol, 224g/mol) was dissolved in DMF and added to a solution containing HOBt 74.9mg (0.49mmol, 153g/mol), HBTU 185.7mg (0.49mmol, 379g/mol) and DIPEA 174 µL (1mmol, 129g/mol, Density 0.74) in DMF. This was added to the H_2N-$PEG_{(2000)}$-GFLG-P18-◕ and agitated on the shaker for 3 hours. Reaction was monitored by Kaiser test.

Mass Spectra results: MALDI-TOF: 5052.4160 g/mol Exact mass: 5053 g/mol

(Appendix 12,12a)

Addition of Hydrazide group

2mL of hydrazine hydrate and 2mL of 1,4 dioxane was placed in the reaction vessel with the pre swollen S-benzyl- $C_4O_2H_4$-NH-$PEG_{(2000)}$-GFLG-P18-◕ in DCM. The reaction was agitated on a shaker for 2 hour. The resin was then washed 3 x 5mins in DCM and the reaction was repeated to ensure the complete removal of the benzyl mercaptan group. (Appendix 13,13a)

Mass Spectra results: MALDI-TOF: 4933.0936 g/mol Exact Mass: 4933g/mol

Cleavage from Solid Support

The peptide was cleaved from the resin in a cleavage cocktail solution, 5100µL TFA, 150µL EDT, 150µL TA, 300µL water, 300µL TIPS, stirring for 2.5 hours. The product was precipitated in diethyl ether as a white solid and collected by centrifugation and washed twice more with diethyl ether. The isolated peptide was dissolved in water and lyophilized overnight and a white powder was collected.

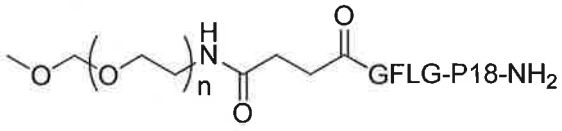
Size exclusion chromatography: 73.622 % pure, Retention Time: 22min 61sec

Addition of DOX

10mg of H_3N-NH-$C_4O_2H_4$-NH-PEG_{2000}-GFLG-P18-NH2 5024g/mol, 0.00199mmol was added into a round bottom flask with DOX.HCl 11.5 mg (579g/mol, 0.0199mmol) and was left stirring for 24hours in anhydrous methanol under argon away from the light. The reaction was monitored by SEC-HPLC. The methanol was then removed by rotary evaporator and placed on a high vacuum line to dry completely. The product was then placed on sephadex LH-20 and the DOX-PEG-P18 was separated out from the excess free DOX. The separation was monitored by SEC HPLC using PBS Buffer at 0.5mL/min, monitoring PDA, at wave lengths 480nm and 214nm for DOX and the peptide amide bond respectively. (Appendix 14)

SEC-Biosep S-2000 HPLC Results: 61.1% pure, Retention Time: 18 min 92 sec

5.2.1.11 Preparation of PEGylated peptide MeO-PEG$_{(5000)}$-GFLG-P18



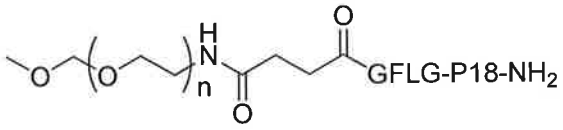
n= 109 number of repeating PEG units

PEGylation of peptide H-GFLG-P18-NH-◕ (**compound 5.2.1.8**) was performed on the solid support by manual coupling. 1000mgs of MeO-PEG$_{(5000)}$-COOH (2000g/mol, 0.2mmol) was added to 1.9 equivalents of HATU 72.0mg and 4 equivalents of DIPEA 69.6 µL in DMF. The solution was then added to the peptide H-GFLG-P18-NH-◕ and left to agitate on a shaker for 3 hours. Coupling was monitored by Kaiser test to ensure complete coupling. The peptide was cleaved from the resin in a cleavage cocktail solution, 5100µL TFA, 150µL EDT, 150µL TA, 300µL water and 300µL TIPS, stirring for 2.5 hours. The product was precipitated in diethyl ether as a white solid and collected by centrifugation. The isolated peptide was dissolved in water and lyophilized. The PEGylated peptide was purified by sephadex G-25 with de-ionized water as the mobile phase. TLC staining by iodine crystals was used to show the presence of PEG and the purity of the product was analyzed by SEC HPLC, Biosep S-2000. A small amount of the sample of dissolved in water and prepared for mass spectrometry MALDI-TOF analysis using α-cyano-4-hydroxycinnamic acid (dissolved in water 0.1% TFA / acetonitrile 0.1% TFA). 5:1 ratio matrix to product. (Appendix 15, 16)

Mass Spectra results: MALDI-TOF 7745.1074g/mol (Appendix 16, 17)

SEC-Biosep S-2000 HPLC Results: 99.5 % pure, Retention Time: 29.27 min

5.2.1.12 Preparation of PEGylated peptide; MeO-PEG$_{(2000)}$-GFLG-P18



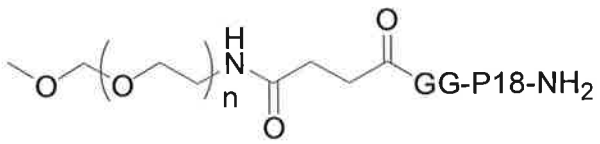
n= 41 repeating PEG units

PEGylation of peptide H-GFLG-P18-NH-◕ (**compound 5.2.1.8**) was performed on the solid support by manual coupling. 400mgs of MeO-PEG$_{(2000)}$-COOH (2000g/mol, 0.2mmol) was added to 1.9 equivalents of HATU 72.0mg and 4 equivalents of DIPEA 69.6 μL in DMF. The solution was then added to the peptide H-GFLG-P18-NH-◕ and left to agitate on a shaker for 3 hours. Coupling was monitored by Kaiser test to ensure complete coupling. The peptide was cleaved from the resin in a cleavage cocktail solution, 5100μL TFA, 150μL EDT, 150μL TA, 300μL water and 300μL TIPS, stirring for 2.5 hours. The product was precipitated in diethyl ether as a white solid and collected by centrifugation. The isolated peptide was dissolved in water and lyophilized. The PEGylated peptide was purified by sephadex G-25 with de-ionized water as the mobile phase. TLC staining by iodine crystals was used to show the presence of PEG and the purity of the product. The product was analyzed by RP-HPLC, semi-prep C5 Jupiter column. A small amount of the sample of dissolved in water and prepared for mass spectrometry MALDI-TOF analysis using α-cyano-4-hydroxycinnamic acid (dissolved in water 0.1% TFA / acetonitrile 0.1% TFA). 5:1 ratio matrix to product. (Appendix 18,19)

Mass Spectra results: MALDI-TOF: 4782.0498 g/mol

RP-HPLC C5 Jupiter Results: 97.02 % pure, Retention Time: 28 min 23 sec

5.2.1.13 Preparation of PEGylated peptide MeO-PEG$_{(2000)}$-GG-P18



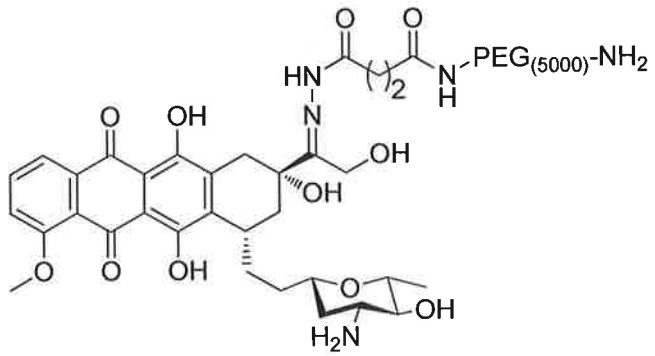
n= 41 number of repeating PEG units

PEGylation of peptide H-GG-P18-NH-◕ (**compound 5.2.1.9**) was performed on the solid support by manual coupling. 400mgs of MeO-PEG$_{(5000)}$-COOH (2000g/mol, 0.2mmol) was added to 1.9 equivalents of HATU 72.0mg and 4 equivalents of DIPEA 69.6 µL in DMF. The solution was then added to the peptide H-GG-P18-NH-◕ and left to agitate on a shaker for 3 hours. Coupling was monitored by Kaiser test to ensure complete coupling. The peptide was cleaved from the resin in a cleavage cocktail solution, 5100µL TFA, 150µL EDT, 150µL TA, 300µL water, 300µL TIPS, stirring for 2.5 hours. The product was precipitated in diethyl ether as a white solid and collected by centrifugation. The isolated peptide was dissolved in water and lyophilized. The PEGylated peptide was purified by sephadex G-25 with de-ionized water as the mobile phase. TLC staining by iodine crystals was used to show the presence of PEG and the purity. The product was analyzed by RP-HPLC, semi-prep C5 Jupiter column. A small amount of the sample of dissolved in water and prepared for mass spectrometry MALDI-TOF analysis using α-cyano-4-hydroxycinnamic acid (dissolved in water 0.1% TFA / acetonitrile 0.1% TFA). 5:1 ratio matrix to product. Appendix (19, 20)

Mass Spectra results: MALDI-TOF: 4465.8735 g/mol

RP-HPLC C5 Jupiter Results: 98.51% pure, Retention Time: 28min 36sec

5.2.1.14 Preparation of PEGylated doxorubicin; DOX-PEG$_{(5000)}$-NH$_2$



Preparation for the PEGylation of DOX was carried on the solid support, rink amide PEGA resin with a substitution of 0.33mmol, 1.69g of wet resin, 0.1mmol scale by manual coupling. 1000mgs of MeO-PEG$_{(5000)}$-COOH (5000g/mol, 0.2mmol) was added to 1.9 equivalents of HATU 72.0mg and 4 equivalents of DIPEA 69.6 µL in DMF. The solution was then added to the resin and left to agitate on a shaker for 3 hours. Coupling was monitored by Kaiser test to ensure complete coupling. Deprotection was carried out on the Fmoc protecting group using the standard deprotection protocol and again monitored by Kaiser test to ensure complete deprotection of the Fmoc group.

Addition of S-benzyl thiosuccinic acid

S-benzyl thiosuccinic acid (**Compound 5.2.1.1**) 112.5mg, (0.5mmol, 224g/mol) was coupled on solid support to the NH$_2$-PEG$_{(5000)}$-C(O)NH-◕ in a solution of HOBt 74.9mg (0.49mmol, 153g/mol), HBTU 185.7mg (0.49mmol, 379g/mol) and DIPEA 174 µL (1mmol, 129g/mol, Density 0.74) in DMF and agitated on the shaker for 3 hours. Reaction was monitored by Kaiser test.

Addition of Hydrazine Group

2mL of hydrazine hydrate and 2mL of 1,4 dioxane was placed in the reaction vessel with the pre swelled S-benzyl-succinic-NH-PEG$_{(5000)}$- C(O)NH-◕ in DCM. The reaction was agitated on a shaker for 2 hour. The resin was then washed 3 x 5mins

in DCM and the reaction was repeated to make sure the removal of the thiobenzyl protecting group.

PEG cleavage from Resin

The PEG-hydrazide was cleaved from the resin in a cleavage cocktail solution, 5700µL, 300 µL DCM and left stirring for 2.5 hours. The TFA/DCM was evaporated in a stream of N_2 and the product was dissolved in water and lyophilized overnight.

Addition of DOX

221mg of PEG-hydrazide (4811g/mol, 0.049mmol), mmol was added into a round bottom flask with DOX.HCl 53mg, (543g/mol, 0.092mmol) with a few drops of acetic acid and left stirring for 24hours with anhydrous methanol under argon in the dark. The methanol was then removed by rota evaporator and placed on a high vacuum line to dry completely. The PEG-DOX was purified on sephadex LH-20 with methanol as the mobile phase. The separation was monitored by SEC HPLC using PBS Buffer pH 6.95 at 0.5mL/min, monitoring at wave lengths 480and 214 for DOX and the peptide amide bond respectively. The DOX conjugation was also confirmed by UV-VIS spectrometry.

RP-HPLC SEC-Biosep S-2000 Results: 96.46 % pure, Retention Time: 13 min 51 sec (Appendix 21)

5.3 Experimental procedure for Chapter 2

5.3.1 Cell viability assay of PEGylated compounds

5.3.1.1 MTS cell viability assay on ovarian cancer cell line A2780p; P18

Cells were seeded at a density of 5,000 cells per well in 96-well plates, in a volume of 150µL of media per well. The plates were set up for the time point at 72hrs. The following day, media was aspirated from cells and replaced with media containing the peptide P18 at concentrations, serial diluted from 100 µM to 0.78 µM. 15µL of MTS was added to the plates after 72hours and left to incubate for 3 hours. The plate was read on a Perkin Elmer plate reader at Abs 490 nm for 1 sec. The assay was performed in triplicate and statistical analysis carried out.

IC_{50} value= 2.2 µM

5.3.1.2 MTS cell viability assay on ovarian cancer cell line A2780p; DOX

Cells were seeded at a density of 5,000 cells per well in 96-well plates, in a volume of 150µL of media per well. The following day, media was aspirated from cells and replaced with media containing the DOX at concentrations, serial diluted from 1 µM to 7.8nM. After 72hours, 15µL of MTS was added to the plate and left to incubate for 3 hours. The plate was read on a Perkin Elmer plate reader at Abs 490 nm for 1 sec. The assay was performed in triplicate and statistical analysis carried out.

IC_{50} value= 98 nM

5.3.1.3 MTS cell viability assay on ovarian cancer cell line A2780p; MeO-PEG$_{(2000)}$-GFLG-P18

Cells were seeded at a density of 5,000 cells per well in 96-well plates, in a volume of 150µL of media per well. The following day, media was aspirated from cells and replaced with media containing the MeO-PEG$_{(2000)}$-GFLG-P18 at concentrations, serial diluted from 100 µM to 0.78µM. After 72hours, 15µL of MTS was added to the plates and left to incubate for 3 hours. The plate was read on a Perkin Elmer plate reader at Abs 490 nm for 1 sec. The assay was performed in triplicate and statistical analysis carried out.

IC_{50} value= 6.8 µM

5.3.1.4 MTS cell viability assay on ovarian cancer cell line A2780p; H-PEG$_{(5000)}$-NH_2

Cells were seeded at a density of 5,000 cells per well in 96-well plates, in a volume of 150µL of media per well. The following day, media was aspirated from cells and replaced with media containing the H-PEG$_{(5000)}$-NH_2 at concentrations, serial diluted from 100 µM to 0.78µM. After 72hours, 15µL of MTS was added to the plates and left to incubate for 3 hours. The plate was read on a Perkin Elmer plate reader at Abs 490 nm for 1 sec. The assay was performed in triplicate and statistical analysis carried out.

IC_{50} value= 29.8 µM

5.3.1.5 MTS cell viability assay on ovarian cancer cell line A2780p; MeO-PEG$_{(2000)}$-GG-P18

Cells were seeded at a density of 5,000 cells per well in 96-well plates, in a volume of 150µL of media per well. The following day, media was aspirated from cells and replaced with media containing the MeO-PEG$_{(2000)}$-GG-P18 at concentrations, serial diluted from 100 µM to 0.78µM. After 72hours, 15µL of MTS was added to the

plates and left to incubate for 3 hours. The plate was read on a Perkin Elmer plate reader at Abs 490 nm for 1 sec. The assay was performed in triplicate and statistical analysis carried out.

IC_{50} value= 87 μM

5.3.1.6 MTS cell viability assay, mouse mammary adenocarcinoma (breast cancer) cells (4T1.2 Luc); P18

Cells were seeded at a density of 10,000 cells per well in 96-well plates, in a volume of 150μL of media per well. 3 plates were set up for 3 different time points at 24, 48 and 72hrs. The following day, media was aspirated from cells and replaced with media containing the peptide P18 at concentrations, serial diluted from 100 μM to 0.78 μM. After 72hours, 15μL of MTS was added to the plates and left to incubate for 3 hours. The plate was read on a Perkin Elmer plate reader at Abs 490 nm for 1 sec. The assay was performed in triplicate and statistical analysis carried out.

IC_{50} value= 16 μM

5.3.1.7 MTS cell viability assay, mouse mammary adenocarcinoma (breast cancer) cells (4T1.2 Luc); MeO-$PEG_{(5000)}$-GFLG-P18

Cells were seeded at a density of 10,000 cells per well in 96-well plates, in a volume of 150μL of media per well. The following day, media was aspirated from cells and replaced with media containing the MeO-$PEG_{(5000)}$-GFLG-P18 at concentrations, serial diluted from 100 μM to 0.78μM. After 72hours, After 72hours, 15μL of MTS was added to the plates and left to incubate for 3 hours. The plate was read on a Perkin Elmer plate reader at Abs 490 nm for 1 sec. The assay was performed in triplicate and statistical analysis carried out.

IC_{50} value= 59 μM

5.3.2 Combination study of PEGylated Compounds

5.3.2.1 MTS cell proliferation assay on ovarian cancer cell line A2780p; DOX-$PEG_{(5000)}$-NH_2 + MeO-$PEG_{(2000)}$-GFLG-P18

Cells were seeded at a density of 5,000 cells per well in 96-well plates, in a volume of 150µL of media per well. The following day, media was aspirated from cells and replaced with media containing 75µL of MeO-$PEG_{(2000)}$-GFLG-P18 and at concentrations, serial diluted from 200 µM to 1.56µM. 75 µL DOX-$PEG_{(5000)}$-NH_2 was added at double the IC_{50} concentration 59.6 µM. After 72hours, 15µM of MTS was added to the different plates at their different time points and left to incubate for 3 hours. The plate was read on a Perkin Elmer plate reader at Abs 490 nm for 1 sec. The assay was performed in triplicate and statistical analysis carried out.

IC_{50} value= 2.93 µM

5.3.2.2 MTS cell proliferation assay on ovarian cancer cell line A2780p; DOX-$PEG_{(5000)}$-NH_2 + MeO-$PEG_{(2000)}$-GFLG-P18

Cells were seeded at a density of 5,000 cells per well in 96-well plates, in a volume of 150µL of media per well. The following day, media was aspirated from cells and replaced with media containing 75µL of DOX-$PEG_{(5000)}$-NH_2 and at concentrations, serial diluted from 2 µM to 15.6nM. 75 µL MeO-$PEG_{(2000)}$-GFLG-P18 was added at double the IC_{50} concentration 13.6 µM. After 72hours, 15µM of MTS was added to the different plates at their different time points and left to incubate for 3 hours. The plate was read on a Perkin Elmer plate reader at Abs 490 nm for 1 sec. The assay was performed in triplicate and statistical analysis carried out.

IC_{50} value= 6.5 µM **FIC=** 0.65 Synergistic/additive effect= Synergistic

5.3.3 MTT Cell viability assays on ovarian cancer cells SK-OV-3 and Hs832 ovarian non-malignant cells

(Cell testing of these assays where performed in collaboration with IT Tallaght by Dr. Agnieska Folytyn-Arfa Kiab and Dr. Siobhan McClean)

5.3.3.1 MTT cell viability assay; Ovarian cancer cell line SK-OV-3 MeO-PEG(5000)-GFLG-P18

Cells were seeded at a density of 40,000 cells per well in 96-well plates, in a volume of 150µL of media per well. The following day, media was aspirated from cells and replaced with media containing MeO-$PEG_{(5000)}$-GFLG-P18 at concentrations, serial diluted from 100 µM to 0.78µM. After 24hours, 20 µM of MTT 5mg/ml in 0.1M PBS pH 7.4 was added to the plates and left to incubate for 4 hours. Media was gently aspirated from the cells and 100 µM of DMSO was added to the wells. The plate was read on a Perkin Elmer plate reader at Abs 550 nm for 1 sec in a Varioscan plate reader. The assay was repeated in triplicate and statistical analysis carried out.

IC_{50} value= 65.3 µM (±15.9)

5.3.3.2 MTT cell viability assay on ovarian cancer cell line SK-OV-3; MeO-$PEG_{(2000)}$-GFLG-P18

Cells were seeded at a density of 40,000 cells per well in 96-well plates, in a volume of 150µL of media per well. The following day, media was aspirated from cells and replaced with media containing MeO-$PEG_{(2000)}$-GFLG-P18 at concentrations, serial diluted from 100 µM to 0.78µM. After 24hours, 20 µM of MTT 5mg/ml in 0.1M PBS pH 7.4 was added to the plates and left to incubate for 4 hours. Media was gently aspirated from the cells and 100 µM of DMSO was added to the wells. The plate was read on a Perkin Elmer plate reader at Abs 550 nm for 1 sec in a Varioscan

plate reader. The assay was repeated in triplicate and statistical analysis carried out.

IC_{50} value= 39.4 μM (±4.2)

5.3.3.3 MTT cell viability assay on ovarian cancer cell line SK-OV-3; MeO-$PEG_{(5000)}$-GG-P18

Cells were seeded at a density of 40,000 cells per well in 96-well plates, in a volume of 150μL of media per well. The following day, media was aspirated from cells and replaced with media containing MeO-$PEG_{(5000)}$-GG-P18 at concentrations, serial diluted from 100 μM to 0.78μM. After 24hours, 20 μM of MTT 5mg/ml in 0.1M PBS pH 7.4 was added to the plates and left to incubate for 4 hours. Media was gently aspirated from the cells and 100 μM of DMSO was added to the wells. The plate was read on a Perkin Elmer plate reader at Abs 550 nm for 1 sec in a Varioscan plate reader. The assay was repeated in triplicate and statistical analysis carried out.

IC_{50} value= 77.9 μM

5.3.3.4 MTT cell viability assay; Ovarian cancer cell line SK-OV-3 DOX-$PEG_{(5000)}$-NH_2

Cells were seeded at a density of 40,000 cells per well in 96-well plates, in a volume of 150μL of media per well. The following day, media was aspirated from cells and replaced with media containing DOX-$PEG_{(5000)}$-NH_2 at concentrations, serial diluted from 100 μM to 0.78μM. After 24hours, 20 μM of MTT 5mg/ml in 0.1M PBS pH 7.4 was added to the plates and left to incubate for 4 hours. Media was gently aspirated from the cells and 100 μM of DMSO was added to the wells. The plate was read on a Perkin Elmer plate reader at Abs 550 nm for 1 sec in a Varioscan plate reader. The assay was repeated in triplicate and statistical analysis carried out.

IC_{50} value= >100 μM

5.3.3.5 MTT cell viability assay on ovarian cancer cell line SK-OV-3 P18

Cells were seeded at a density of 40,000 cells per well in 96-well plates, in a volume of 150µL of media per well. The following day, media was aspirated from cells and replaced with media containing P18 at concentrations, serial diluted from 100 µM to 0.78µM. After 24hours, 20 µM of MTT 5mg/ml in 0.1M PBS pH 7.4 was added to the plates and left to incubate for 4 hours. Media was gently aspirated from the cells and 100 µM of DMSO was added to the wells. The plate was read on a Perkin Elmer plate reader at Abs 550 nm for 1 sec in a Varioscan plate reader. The assay was repeated in triplicate and statistical analysis carried out.

IC_{50} value= 17.4 µM (±10)

5.3.3.6 MTT cell viability assay on ovarian cancer cell line SK-OV-3; DOX

Cells were seeded at a density of 40,000 cells per well in 96-well plates, in a volume of 150µL of media per well. The following day, media was aspirated from cells and replaced with media containing DOX at concentrations, serial diluted from 100 µM to 0.78µM. After 24hours, 20 µM of MTT 5mg/ml in 0.1M PBS pH 7.4 was added to the plates and left to incubate for 4 hours. Media was gently aspirated from the cells and 100 µM of DMSO was added to the wells. The plate was read on a Perkin Elmer plate reader at Abs 550 nm for 1 sec in a Varioscan plate reader. The assay was repeated in triplicate and statistical analysis carried out.

IC_{50} value= 68.3 µM (±25)

5.3.3.7 MTT cell viability assay on Hs832 ovarian non-malignant cells; MeO-$PEG_{(5000)}$-GFLG-P18

Cells were seeded at a density of 40,000 cells per well in 96-well plates, in a volume of 150µL of media per well. The following day, media was aspirated from cells and replaced with media containing MeO-PEG$_{(5000)}$-GFLG-P18 at concentrations, serial diluted from 100 µM to 0.78µM. After 24hours, 20 µM of MTT 5mg/ml in 0.1M PBS pH 7.4 was added to the plates and left to incubate for 4 hours. Media was gently aspirated from the cells and 100 µM of DMSO was added to the wells. The plate was read on a Perkin Elmer plate reader at Abs 550 nm for 1 sec in a Varioscan plate reader. The assay was repeated in triplicate and statistical analysis carried out.

IC_{50} value= 42.3 µM (±2.1)

5.3.3.8 MTT cell viability assay on Hs832 ovarian non-malignant cells; MeO-PEG$_{(2000)}$-GFLG-P18

Cells were seeded at a density of 40,000 cells per well in 96-well plates, in a volume of 150µL of media per well. The following day, media was aspirated from cells and replaced with media containing MeO-PEG$_{(2000)}$-GFLG-P18 at concentrations, serial diluted from 100 µM to 0.78µM. After 24hours, 20 µM of MTT 5mg/ml in 0.1M PBS pH 7.4 was added to the plates and left to incubate for 4 hours. Media was gently aspirated from the cells and 100 µM of DMSO was added to the wells. The plate was read on a Perkin Elmer plate reader at Abs 550 nm for 1 sec in a Varioscan plate reader. The assay was repeated in triplicate and statistical analysis carried out.

IC_{50} value= 19.0 µM (±5.3)

5.3.3.9 MTT cell viability assay on Hs832 ovarian non-malignant cells; MeO-PEG$_{(5000)}$-GG-P18

Cells were seeded at a density of 40,000 cells per well in 96-well plates, in a volume of 150µL of media per well. The following day, media was aspirated from cells and

replaced with media containing MeO-$PEG_{(5000)}$-GG-P18 at concentrations, serial diluted from 100 µM to 0.78µM. After 24hours, 20 µM of MTT 5mg/ml in 0.1M PBS pH 7.4 was added to the plates and left to incubate for 4 hours. Media was gently aspirated from the cells and 100 µM of DMSO was added to the wells. The plate was read on a Perkin Elmer plate reader at Abs 550 nm for 1 sec in a Varioscan plate reader. The assay was repeated in triplicate and statistical analysis carried out.

IC_{50} value= 51.8 µM (±9.8)

5.3.3.10 MTT cell viability assay on Hs832 ovarian non-malignant cells; DOX-$PEG_{(5000)}$-NH_2

Cells were seeded at a density of 40,000 cells per well in 96-well plates, in a volume of 150µL of media per well. The following day, media was aspirated from cells and replaced with media containing DOX-$PEG_{(5000)}$-NH_2 at concentrations, serial diluted from 100 µM to 0.78µM. After 24hours, 20 µM of MTT 5mg/ml in 0.1M PBS pH 7.4 was added to the plates and left to incubate for 4 hours. Media was gently aspirated from the cells and 100 µM of DMSO was added to the wells. The plate was read on a Perkin Elmer plate reader at Abs 550 nm for 1 sec in a Varioscan plate reader. The assay was repeated in triplicate and statistical analysis carried out.

IC_{50} value= >100

5.3.3.11 MTT cell viability assay on Hs832 ovarian non-malignant cells; P18

Cells were seeded at a density of 40,000 cells per well in 96-well plates, in a volume of 150µL of media per well. The following day, media was aspirated from cells and

replaced with media containing P18 at concentrations, serial diluted from 100 µM to 0.78µM. After 24hours, 20 µM of MTT 5mg/ml in 0.1M PBS pH 7.4 was added to the plates and left to incubate for 4 hours. Media was gently aspirated from the cells and 100 µM of DMSO was added to the wells. The plate was read on a Perkin Elmer plate reader at Abs 550 nm for 1 sec in a Varioscan plate reader. The assay was repeated in triplicate and statistical analysis carried out.

IC_{50} value= 5.8 µM (±1.0)

5.3.3.12 MTT cell viability assay on Hs832 ovarian non-malignant cells; DOX

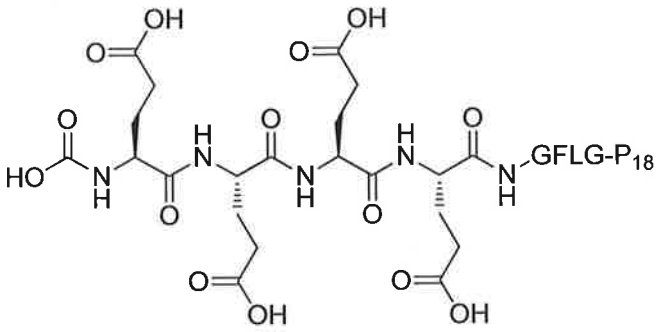
Cells were seeded at a density of 40,000 cells per well in 96-well plates, in a volume of 150µL of media per well. The following day, media was aspirated from cells and replaced with media containing DOX at concentrations, serial diluted from 1 µM to 0.19nM. After 24hours, 20 µM of MTT 5mg/ml in 0.1M PBS pH 7.4 was added to the plates and left to incubate for 4 hours. Media was gently aspirated from the cells and 100 µM of DMSO was added to the wells. The plate was read on a Perkin Elmer plate reader at Abs 550 nm for 1 sec in a Varioscan plate reader. The assay was repeated in triplicate and statistical analysis carried out.

IC_{50} value= >100 µM

5.4 Experimental procedure for Chapter 3

5.4.1 Synthesis of Polyglutamic Peptide Prodrugs

5.4.1.1 Preparation of Peptide; Ac-EEEE-GFLG-P18



Peptide H-GFLG-P18, 0.1mmol, was synthesised as described in **compound 5.2.1.8.** The addition of the polyglutamic acid was added by manual synthesis. PyBop/HOBt/DIPEA was used to couple the Fmoc-D-Glu(O*t*Bu)-OH. The coupling reaction was performed using 5 equivalents of coupling reagents with PyBOP/HOBt, 260mg (0.1mmol, 520g/mol), 76.5mg (0.1mmol, 153g/mol), and 10 equivalents of DIPEA 174µL (1.0mmol, 129g/mol, density 0.74) dissolved in DMF and added to 5 equivalents of Fmoc-D-Glu(OtBu)-OH, 212.5mg (0.1mmol, 425g/mol). The solution was then added to the peptide H-GFLG-P18-◕ and left to agitate on a shaker for 3 hours. Coupling was monitored by Kaiser test to ensure complete coupling. Deprotection was carried out on the Fmoc protecting group using the standard deprotection protocol and again monitored by the Kaiser test to ensure complete deprotection of the Fmoc group. This process cycle was repeated 4 times and the N-terminus was capped using acetic acid. Acetic acid 57µL (60.09g/mol, density 0.74) was coupled in the same way as above. The peptide was cleaved from the resin in a cleavage cocktail solution, 5100µL TFA, 150µL EDT, 150µL TA, 300µL water, 300µL TIPS, stirring for 2.5 hours. The product was precipitated in diethyl ether as a white solid and collected by centrifugation, washed twice more with diethyl ether. The isolated peptide was dissolved in water and lyophilized overnight

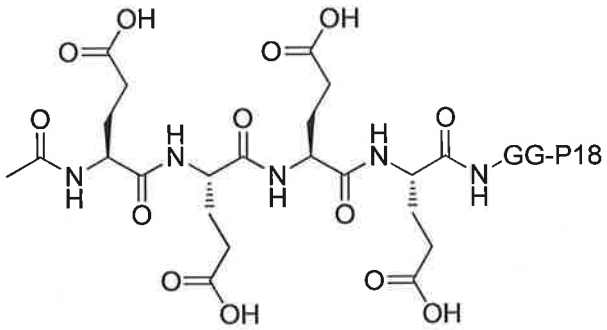
and a white powder was collected. The product was then purified by RP-HPLC, semi-prep C18 Gemini column. 1mg of the pure sample was taken for mass spectrometry MALDI-TOF analysis using α-cyano-4-hydroxycinnamic acid. The purity of the peptide was analysed by RP-HPLC, analytical C18 Gemini column.

Mass Spectra results: Exact Mass:3232.906 MALDI-TOF 3231.5193 g/mol

RP-HPLC C18 Gemini Results: 98.05% pure, Retention Time: 24 min 32 sec

(Appendix 22, 23)

5.4.1.2 Preparation of Peptide; Ac-EEEE-GG-P18



Peptide H-GFLG-P18, 0.1mmol, was synthesised as described in **compound 5.2.1.9.** The addition of the polyglutamic acid was added by manual synthesis. PyBop/HOBt/DIPEA was used to couple the Fmoc-D-Glu(OtBu)-OH. The coupling reaction was performed using 5 equivalents of coupling reagents with PyBOP/HOBt, 260mg (0.1mmol, 520g/mol), 76.5mg (0.1mmol, 153g/mol), and 10 equivalents of DIPEA 174μL (1.0mmol, 129g/mol, density 0.74) dissolved in DMF and added to 5 equivalents of Fmoc-D-Glu(OtBu)-OH, 212.5mg (0.1mmol, 425g/mol). The solution was then added to the peptide H-GG-P18-◕ and left to agitate on a shaker for 3 hours. Coupling was monitored by Kaiser test to ensure complete coupling. Deprotection was carried out on the Fmoc protecting group using the standard deprotection protocol and again monitored by Kaiser test to ensure complete

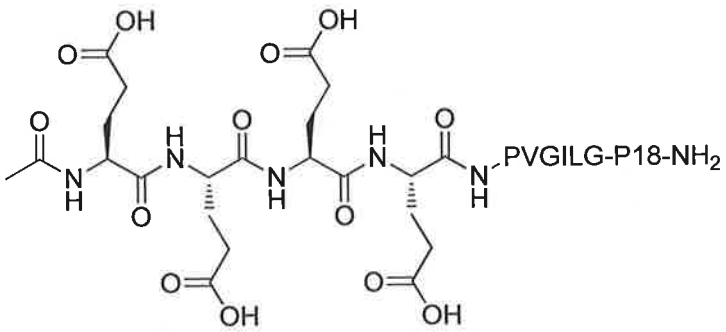
deprotection of the Fmoc group. This process cycle was repeated 4 times and the N-terminus was capped using acetic acid. Acetic acid 57µL (60.09g/mol, density 0.74) was coupled in the same way as above. The peptide was cleaved from the resin in a cleavage cocktail solution, 5100µL TFA, 150µL EDT, 150µL TA, 300µL water, 300µL TIPS, stirring for 2.5 hours. The product was precipitated in diethyl ether as a white solid and collected by centrifugation, washed twice more with diethyl ether. The isolated peptide was dissolved in water and lyophilized overnight and a white powder was collected. The product was then purified by RP-HPLC, semi-prep C18 Gemini column. 1mg of the pure sample was taken for mass spectrometry MALDI-TOF analysis using α-cyano-4-hydroxycinnamic acid. The purity of the peptide was analysed by RP-HPLC, analytical C18 Gemini column.

Mass Spectra results: Exact Mass: 2971.570 MALDI-TOF: 2971.1687g/mol

RP-HPLC C18 Gemini Results: 99.07 % pure, Retention Time: 23min 31sec

(Appendix 24,25)

5.4.1.3 Preparation of Peptide; Ac-EEEE-PVGILG-P18



Peptide H-P18, 0.1mmol, was synthesised as described in **compound 5.2.1.7.** The heptapeptide linker PVGILG (MMP-2) was also added on the synthesizer but double coupled (20equivalents) after the Lys[1] on all of the amino acids on the MMP-2 linker. The polyglutamic acid was added by manual synthesis. HATU/DIPEA was

used to couple the Fmoc-D-Glu(OtBu)-OH. The coupling reaction was performed using 10 equivalents of coupling reagents with HATU 380mg (0.1mmol, 380g/mol) and 20 equivalents of DIPEA 348μL (2.0mmol, 129g/mol, density 0.74) dissolved in DMF and added to 10 equivalents of Fmoc-D-Glu(OtBu)-OH, 425mg (0.5mmol, 425g/mol). The solution was then added to the peptide H-PVGILG-P18-◕ and left to agitate on a shaker for 3 hours. Coupling was monitored by the Kaiser test to ensure complete coupling. Deprotection was carried out on the Fmoc protecting group using the standard deprotection protocol and again monitored by the Kaiser test to ensure complete deprotection of the Fmoc group. This process was repeated 4 times and the N-terminus was capped using acetic acid. Acetic acid 57μL (60.09g/mol, density 0.74) was added to a solution with 10 equivalents of HATU 380mg (0.1mmol, 380g/mol) and 20 equivalents of DIPEA 348 μL (0.76mmol, 129g/mol, Density 0.74) in DMF. The solution was then added to the peptide H-EEEE-PVGILG-P18-◕ and left to agitate on a shaker for 3 hours. The peptide was cleaved from the resin in a cleavage cocktail solution, 5100μL TFA, 150μL EDT, 150μL TA, 300μL water, 300μL TIPS, stirring for 2.5 hours. The product was precipitated in diethyl ether as a white solid and collected by centrifugation and washed twice more with diethyl ether. The isolated peptide was dissolved in water and lyophilized overnight and a white powder was collected. The product was purified by RP-HPLC, semi-prep C18 Gemini column. 1mg of the pure sample was taken for mass spectrometry MALDI-TOF analysis using α-cyano-4-hydroxycinnamic acid. The purity of the peptide was analysed by RP-HPLC, analytical C18 Gemini column.

Mass Spectra results: Exact Mass: 3393.138 MALDI-TOF: 3393.5999 g/mol

RP-HPLC C18 Gemini Results: 97.96 % pure, Retention Time: 24 min 35sec

(Appendix 26,27)

5.4.2 Cell viability assay for polyglutamic acid compounds

5.4.2.1 MTS cell viability assay on ovarian cancer cell line A2780p; Ac-EEEE -GFLG-P18

Cells were seeded at a density of 5,000 cells per well in 96-well plates, in a volume of 150µL of media per well. The following day, media was aspirated from cells and replaced with media containing the Ac-EEEE-GFLG-P18 at concentrations, serial diluted from 1 µM to 7.8nM. After 72hours, 15µM of MTS was added to the plates and left to incubate for 3 hours. The plate was read on a Perkin Elmer plate reader at Abs 490 nm for 1 sec. The assay was performed in triplicate and statistical analysis carried out.

IC_{50} value= 16.9 µM

5.4.2.2 MTS cell viability assay on ovarian cancer cell line A2780p; Ac-EEEE-GG-P18

Cells were seeded at a density of 5,000 cells per well in 96-well plates, in a volume of 150µL of media per well. The following day, media was aspirated from cells and replaced with media containing the Ac-EEEE-GG-P18 at concentrations, serial diluted from 1 µM to 7.8nM. After 72hours, 15µM of MTS was added to the plates and left to incubate for 3 hours. The plate was read on a Perkin Elmer plate reader at Abs 490 nm for 1 sec. The assay was performed in triplicate and statistical analysis carried out.

IC_{50} value= 33.1 µM

5.4.3 MTS Cell viability on REN (epithelioid-type mesothelioma), Msto-211H (biphasic-type mesothelioma) and H2052

(sacomatoid-type mesothelioma) and cell lines (Cell testing of these assays where performed in collaboration with RCSI ERC Beaumount Hospital by Dr. Warren Thomas)

5.4.3.1 MTS cell proliferation assay on REN (epithelioid-type mesothelioma) cancer cell line; P18

Cells were seeded at a density of 3,000 cells per well in 96-well plates, in a volume of 150µL of media per well. The following day, media was aspirated from cells and replaced with media containing the peptide P18 at concentrations, serial diluted from 40 µM to 0.67 µM. After 24 and 48hours, 15µL of MTS was added to the plates and left to incubate for 3 hours. The plate was read on a Perkin Elmer plate reader at Abs 492 nm for 1 sec. The assay was performed in triplicate and statistical analysis carried out.

IC_{50} value= 2.60 µM

5.4.3.2 MTS cell viability assay on REN (epithelioid-type mesothelioma) cancer cell line; Ac-EEEE-GG-P18

Cells were seeded at a density of 3,000 cells per well in 96-well plates, in a volume of 150µL of media per well. The following day, media was aspirated from cells and replaced with media containing the peptide Ac-EEEE-GG-P18 at concentrations, serial diluted from 40 µM to 0.63 µM. After 48hours, 15µL of MTS was added to the plates and left to incubate for 3 hours. The plate was read on a Perkin Elmer plate reader at Abs 492 nm for 1 sec. The assay was performed in triplicate and statistical analysis carried out.

IC_{50} value= >50µM

5.4.3.3 MTS cell viability assay on REN (epithelioid-type mesothelioma) cancer cell line; Ac-EEEE-PVGLIFG-P18

Cells were seeded at a density of 3,000 cells per well in 96-well plates, in a volume of 150µL of media per well. The following day, media was aspirated from cells and replaced with media containing the peptide Ac-EEEE-PVGLIFG-P18 at concentrations, serial diluted from 40 µM to 0.63 µM. After 48hours, 15µL of MTS was added to the plates and left to incubate for 3 hours. The plate was read on a Perkin Elmer plate reader at Abs 492 nm for 1 sec. The assay was performed in triplicate and statistical analysis carried out.

IC_{50} value= 0.94 µM

5.4.3.4 MTS cell proliferation assay on Msto-211H (biphasic-type mesothelioma) cancer cell line; P18

Cells were seeded at a density of 3,000 cells per well in 96-well plates, in a volume of 150µL of media per well. The following day, media was aspirated from cells and replaced with media containing the peptide P18 at concentrations, serial diluted from 40 µM to 0.63 µM. After 48hours, 15µM of MTS was added to the plates and left to incubate for 3 hours. The plate was read on a Perkin Elmer plate reader at Abs 492 nm for 1 sec. The assay was performed in triplicate and statistical analysis carried out.

IC_{50} value= 1.44 µM

5.4.3.5 MTS cell viability assay on Msto-211H (biphasic-type mesothelioma) cancer cell line; Ac-EEEE-GFLG-P18

Cells were seeded at a density of 3,000 cells per well in 96-well plates, in a volume of 150µL of media per well. The following day, media was aspirated from cells and replaced with media containing the peptide Ac-EEEE-GFLG-P18 at concentrations, serial diluted from 40 µM to 0.63 µM. After 48hours, 15µM of MTS was added to

the plates and left to incubate for 3 hours. The plate was read on a Perkin Elmer plate reader at Abs 492 nm for 1 sec. The assay was performed in triplicate and statistical analysis carried out.

IC_{50} value= 6.69 μM

5.4.3.6 MTS cell viability assay on Msto-211H (biphasic-type mesothelioma) cancer cell line; Ac-EEEE-GG-P18

Cells were seeded at a density of 3,000 cells per well in 96-well plates, in a volume of 150μL of media per well. The following day, media was aspirated from cells and replaced with media containing the peptide Ac-EEEE-GG-P18 at concentrations, serial diluted from 40 μM to 0.63 μM. After 48hours, 15μM of MTS was added to the plates and left to incubate for 3 hours. The plate was read on a Perkin Elmer plate reader at Abs 492 nm for 1 sec. The assay was performed in triplicate and statistical analysis carried out.

IC_{50} value= >50 μM

5.4.3.7 MTS cell viability assay on Msto-211H (biphasic-type mesothelioma) cancer cell line; Ac-EEEE-PVGLIFG-P18

Cells were seeded at a density of 3,000 cells per well in 96-well plates, in a volume of 150μL of media per well. The following day, media was aspirated from cells and replaced with media containing the peptide Ac-EEEE-PVGLIFG-P18 at concentrations, serial diluted from 40 μM to 0.63 μM. After 48hours, 15μM of MTS was added to the plates and left to incubate for 3 hours. The plate was read on a Perkin Elmer plate reader at Abs 492 nm for 1 sec. The assay was performed in triplicate and statistical analysis carried out.

IC_{50} value= 1.33 μM

5.4.3.8 MTS cell proliferation assay on H2052 (sacomatoid-type mesothelioma) cancer cell line; P18

Cells were seeded at a density of 3,000 cells per well in 96-well plates, in a volume of 150µL of media per well. The following day, media was aspirated from cells and replaced with media containing the peptide P18 at concentrations, serial diluted from 40 µM to 0.63 µM. After 48hours, 15µM of MTS was added to the plates and left to incubate for 3 hours. The plate was read on a Perkin Elmer plate reader at Abs 492 nm for 1 sec. The assay was performed in triplicate and statistical analysis carried out.

IC_{50} value= 2.5 µM

5.4.3.9 MTS cell viability assay on H2052 (sacomatoid-type mesothelioma) cancer cell line; Ac-EEEE-GFLG-P18

Cells were seeded at a density of 3,000 cells per well in 96-well plates, in a volume of 150µL of media per well. The following day, media was aspirated from cells and replaced with media containing the peptide Ac-EEEE-GFLG-P18 at concentrations, serial diluted from 40 µM to 0.63 µM. After 48hours, 15µM of MTS was added to the plates and left to incubate for 3 hours. The plate was read on a Perkin Elmer plate reader at Abs 492 nm for 1 sec. The assay was performed in triplicate and statistical analysis carried out.

IC_{50} value= 14.9µM

5.4.3.10 MTS cell viability assay on H2052 (sacomatoid-type mesothelioma) cancer cell line; Ac-EEEE-GG-P18

Cells were seeded at a density of 3,000 cells per well in 96-well plates, in a volume of 150µL of media per well. The following day, media was aspirated from cells and replaced with media containing the peptide Ac-EEEE-GG-P18 at concentrations, serial diluted from 40 µM to 0.63 µM. After 48hours, 15µM of MTS was added to

the plates and left to incubate for 3 hours. The plate was read on a Perkin Elmer plate reader at Abs 492 nm for 1 sec. The assay was performed in triplicate and statistical analysis carried out.

IC_{50} value= 44.9 μM

5.4.3.11 MTS cell viability assay on H2052 (sacomatoid-type mesothelioma) cancer cell line; Ac-EEEE-PVGLIFG-P18

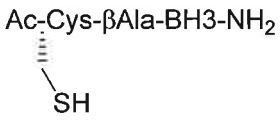
Cells were seeded at a density of 3,000 cells per well in 96-well plates, in a volume of 150μL of media per well. The following day, media was aspirated from cells and replaced with media containing the peptide Ac-EEEE-PVGLIFG-P18 at concentrations, serial diluted from 40 μM to 0.63 μM. After 48hours, 15μM of MTS was added to the plates and left to incubate for 3 hours. The plate was read on a Perkin Elmer plate reader at Abs 492 nm for 1 sec. The assay was performed in triplicate and statistical analysis carried out.

IC_{50} value= 1.76 μM

5.5 Experimental for Chapter 4

5.5.1 Synthesis of Heterodimer Peptides (βAla linker)

5.5.1.1 Synthesis of AC-Cys-βAla-BH3-NH_2

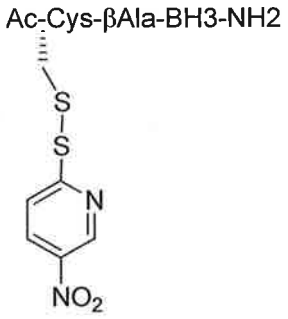


Procedure: The peptide, BH3, was synthesized fom 'L' amino acids, with the sequence H-EDIIRNIARHLAQVGDSMDR-NH_2 (BH3) by Fmoc/*t*Bu strategy on MBHA Rink Amide resin with a substitution of 0.7mmol, 0.143g, 0.1mmol scale by automated peptide synthesis Applied Biosystems 433a. The Fmoc protected amino acids were used with 10 equivalents and double coupled with 20 equivalents at ile[7] until the end of the sequence. The sequence was modified by the addition of a Fmoc-β-Ala-OH linker, Fmoc-Cys(Trt)-OH and acetylated with acetic acid. The peptide was cleaved from the resin in a cleavage cocktail solution, 5100µL TFA, 150µL EDT, 150µL TA, 300µL water, 300µL TIPS, stirring for 4.5 hours under an inert atmosphere. The product was precipitated in diethyl ether as a white solid and collected by centrifugation and washed twice more with diethyl ether. The isolated peptide was dissolved in water and lyophilized overnight and a white powder was collected. The product was purified by RP-HPLC, semi-prep C18 Gemini column. Once collected the peptide was lyophilized overnight. 1mg of the pure sample was taken for mass spectrometry MALDI-TOF analysis using α-cyano-4-hydroxycinnamic acid. The purity of the peptide was analysed by RP-HPLC, analytical C18 Gemini column. (Appendix 28,29)

Mass Spectra results: Exact Mass: 2524.869 g/mol MALDI-TOF: 2524.0650 g/mol

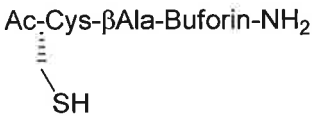
RP-HPLC Results: 87.4 % pure, Retention Time: 27.87

5.5.1.2 Synthesis of AC-Cys(pNpys)-βAla-BH3-NH_2



Procedure: Compound 1.7.1 was dissolved in a stirred solution of DTNP 31mgs (10eq, 310g/mol) in 10mL acetic acid : water (3:1, v/v) and kept under an inert atmosphere. The reaction was monitored by RP-HPLC until complete conversion of the pNpys-Peptide. Acetic acid was then added to reach a proportion of 9:1 v/v and the solvent was eliminated by lyophilization. The product was then extracted with aqueous 0.1% TFA, sonicated, centrifuged and the supernatant freeze-dried. The pNpys-peptide still contained traces of DTNP and they were finally fully removed by washing (5x) the lyophilized powder with diethyl ether:dichloromethane (7:3, v/v) and again freeze dried.

5.5.1.3 Synthesis of AC-Cys-βAla-Buforin-NH_2



Procedure: The peptide, Buforin IIb, was synthesized in "D" amino acids, with the sequence H-raglqfpvgrllrrllrrllr-NH_2 (Buforin IIb) by Fmoc/*t*Bu strategy on MBHA Rink Amide resin with a substitution of 0.7mmol, 0.143g, 0.1mmol scale by Applied Biosystems 433a automated peptide synthesis. The Fmoc protected amino acids

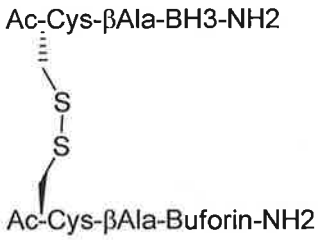
were used with 10 equivalents and double coupling with 20 equivalents from Phe[6] after Pro[7] until the end of the sequence. The sequence was modified by the addition of a Fmoc-β-Ala-OH linker, Fmoc-Cys(Trt)-OH and the N-terminus acetylated with acetic acid. The peptide was cleaved from the resin in a cleavage cocktail solution, 5100µL TFA, 150µL EDT, 150µL TA, 300µL water, 300µL TIPS, stirring for 4.5 hours under an inert atmosphere. The product was precipitated in diethyl ether as a white solid and collected by centrifugation and washed twice more with diethyl ether. The isolated peptide was dissolved in water and lyophilized overnight and a white powder was collected. The product was purified by RP-HPLC, semi-prep C18 Gemini column. Once collected the peptide was lyophilized overnight. 1mg of the pure sample was taken for mass spectrometry MALDI-TOF analysis using α-cyano-4-hydroxycinnamic acid. The purity of the peptide was analysed by RP-HPLC, analytical C18 Gemini column.

Mass Spectra results: Exact Mass: 2776.448 MALDI-TOF: 2776.4080 g/mol

RP-HPLC Results: 81.2% pure, Retention Time: 29.10

Appendix (30,31)

5.5.1.4 Synthesis of Heterodimer peptide



The peptide 1.7.3 was dissolved in a 0.1M acetic acid (pH *ca* 2.7) and the second peptide was added in 1.2 equivalents. The reaction was monitored by RP-HPLC and the conjugation was complete in 6 hours. The isolated peptide was dissolved in water and lyophilized overnight and a white powder was collected. The product was purified by RP-HPLC, semi-prep C18 Gemini column. Once collected the peptide

was lyophilized overnight. 1mg of the pure sample was taken for mass spectrometry MALDI-TOF analysis using α-cyano-4-hydroxycinnamic acid. The purity of the peptide was analysed by RP-HPLC, analytical C18 Gemini column.

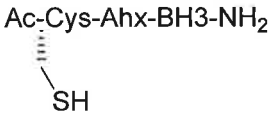
Mass Spectra results: Exact Mass: 5301.320 m/z ESI^+= 1040.60 (z=4)

RP-HPLC Results: 59.8% pure, Retention Time: 27.51

(Appendix 32,33)

5.5.2 Synthesis of Heterodimer Peptides (Ahx linker)

5.5.2.1 Synthesis of AC-Cys-Ahx-BH3-NH_2



Procedure: The peptide, BH3, was synthesized fom 'L' amino acids, with the sequence H-EDIIRNIARHLAQVGDSMDR-NH_2 (BH3) by Fmoc/*t*Bu strategy on MBHA Rink Amide resin with a substitution of 0.7mmol, 0.143g, 0.1mmol scale by automated peptide synthesis Applied Biosystems 433a. The Fmoc protected amino acids were used with 10 equivalents and double coupled with 20 equivalents at ile^7 until the end of the sequence. The sequence was modified by the addition of a Fmoc-Ahx-Ala-OH linker, Fmoc-Cys(Trt)-OH and acetylated with acetic acid. The peptide was cleaved from the resin in a cleavage cocktail solution, 5100µL TFA, 150µL EDT, 150µL TA, 300µL water, 300µL TIPS, stirring for 4.5 hours under an inert atmosphere. The product was precipitated in diethyl ether as a white solid and collected by centrifugation and washed twice more with diethyl ether. The isolated peptide was dissolved in water and lyophilized overnight and a white powder was collected. The product was purified by RP-HPLC, semi-prep C18 Gemini column. Once collected the peptide was lyophilized overnight. 1mg of the pure sample was taken for mass spectrometry MALDI-TOF analysis using α-cyano-4-hydroxycinnamic

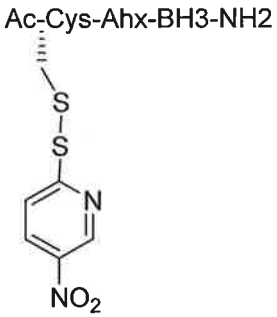
acid. The purity of the peptide was analysed by RP-HPLC, analytical C18 Gemini column.

Mass Spectra results: Exact Mass: 2564.382 ESI^+: 857.16 (m/z=3)

RP-HPLC Results: 58.6% pure, Retention Time: 37.56

(Appendix 34,35)

5.5.2.2 Synthesis of AC-Cys(pNpys)-Ahx-BH3-NH_2



Compound 1.7.4 was dissolved in a stirring solution of DTNP 31mgs (10eq, 310g/mol) in 10mL acetic acid : water (3:1, v/v) and kept under an anhydrous atmosphere. The reaction was monitored by RP-HPLC until complete conversion of the pNpys-Peptide. Acetic acid was then added to reach a proportion of 9:1 v/v and the solvent was eliminated by lyophilization. The product was then extracted with aqueous 0.1% TFA, sonicated, centrifuged and the supernatant freeze-dried. The pNpys-peptide still contained traces of DTNP and they were finally fully removed by washing (5x) the lyophilized powder with diethyl ether:dichloromethane (7:3, v/v) and again freeze dried.

5.5.2.3 AC-Cys-Ahx- Buforin-NH_2

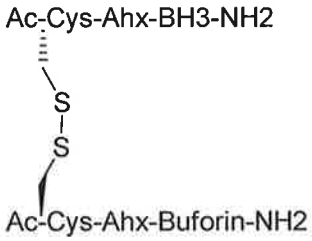
Procedure: The peptide, Buforin IIb, was synthesized in "D" amino acids, with the sequence H-raglqfpvgrllrrllrrllr-NH_2 (Buforin IIb) by Fmoc/*t*Bu strategy on MBHA Rink Amide resin with a substitution of 0.7mmol, 0.143g, 0.1mmol scale by Applied Biosystems 433a automated peptide synthesis. The Fmoc protected amino acids were used with 10 equivalents and double coupling with 20 equivalents from Phe^6 after Pro^7 until the end of the sequence. The sequence was modified by the addition of a Fmoc-Ahx-Ala-OH linker, Fmoc-Cys(Trt)-OH and the N-terminus acetylated with acetic acid. The peptide was cleaved from the resin in a cleavage cocktail solution, 5100µL TFA, 150µL EDT, 150µL TA, 300µL water, 300µL TIPS, stirring for 4.5 hours under an inert atmosphere. The product was precipitated in diethyl ether as a white solid and collected by centrifugation and washed twice more with diethyl ether. The isolated peptide was dissolved in water and lyophilized overnight and a white powder was collected. The product was purified by RP-HPLC, semi-prep C18 Gemini column. Once collected the peptide was lyophilized overnight. 1mg of the pure sample was taken for mass spectrometry MALDI-TOF analysis using α-cyano-4-hydroxycinnamic acid. The purity of the peptide was analysed by RP-HPLC, analytical C18 Gemini column.

Mass Spectra results: Exact Mass: 2816.073 MALDI-TOF: ESI^+: 563.27 (m/z =5)

RP-HPLC Results: 93.6% pure, Retention Time: 29.00

(Appendix 36,37)

5.5.2.4 Synthesis of Heterodimer peptide



Procedure: The peptide 1.7.6 was dissolved in a 0.1M acetic acid (pH *ca* 2.7) and the second peptide 1.7.7 was added in 1.2 equivalents. The reaction was monitored by RP-HPLC and the conjugation was complete in 6 hours. The isolated peptide was dissolved in water and lyophilized overnight and a white powder was collected. The product was purified by RP-HPLC, semi-prep C18 Gemini column. Once collected the peptide was lyophilized overnight. 1mg of the pure sample was taken for mass spectrometry MALDI-TOF analysis using α-cyano-4-hydroxycinnamic acid. The purity of the peptide was analysed by RP-HPLC, analytical C18 Gemini column.

Mass Spectra results: Exact Mass: 5381.076 MALDI-TOF: 5380.1281

RP-HPLC Results: 97.6 % pure, Retention Time: 35.74

(Appendix 38,39)

Chapter 6

Conclusion and future work

6 Conclusion and Future Work

Conclusion and Future Work

The objective of this project was to develop HDP-based candidates to target and combat cancer cells. The project as a whole can be broken down into 3 separate approaches with focuses on different aspects and techniques of drug delivery. The common theme however is the use of prodrugs of HDPs and/or their combinations to meet this objective. This work shows the versatility of HDPs which can be used to target diseases like infection, cancer and inflammatory conditions. Although the focus in this thesis is on targeting cancer cells, the prodrug methodology developed for HDPs has proven to be versatile and extendable to anti-infective applications. This manipulation of the methodology relies however on the identification of molecular mechanisms, in particular enzymatic, specific of the target disease. Collaborative approaches are also critical to these developments to ensure that the depth of knowledge of molecular pathways/surface receptors/proteins/enzymes is captured and that the interpretation of the biological results obtained from prodrugs is performed across multidisciplines.

The first approach within this thesis involved a polymeric peptide prodrug combined with a classical anticancer agent doxorubicin. A dual prodrug approach was developed originally but proved to be synthetically challenging to even sustain *in vitro* studies. It also only offered fixed linearity in the ratio of peptide to DOX (1:1) delivered. From here, a combination prodrug approach was taken and developed. By combining a PEGylated peptide and doxorubicin prodrugs proved to address the limitation of the prior approach. The biological testing was done across a number a different cell lines and showed that the optimum PEG size was of 2000 daltons, to best compromise the requirement of large PEGs for the improvement of the pharmaco-kinetic properties of the peptide and occurrence of the EPR effect, on one hand, and the proteolytic activation by the targeted enzyme, on the other hand. The optimum PEG size *in vivo* would be ultimately determined by the balance between solubility and retention in the body and re-establishment of the activity.

Independently , results obtained with H-PEG_{5000}-DOX-NH_2 showed a significant loss of activity for the classical antineoplastic agent associated with this prodrug, as compared to doxorubicin alone. Going forward a smaller PEG would be more suited for combination studies with the PEGylated peptide prodrug. The results obtained indicate however a synergistic effect between the two agents. Mixing them in the optimal ratio and with the most advantageous PEG sizes could allow this combination to progress to the level of an animal study. Furthermore, the present work inspired the development of PEGylated peptide prodrugs for anti-infective applications. By altering the enzyme degradable peptide linker to a sequence sensitive to a virulence factor of *Staphylococcus aureus*, antistaphylococcal PEGylated peptide prodrug candidates were generated and applicable as targeted antibiotics for the treatment of MRSA infections. These candidates are currently being tested in collaboration with Dr. Deirdre Fitzgerald-Hughes, Beaumont Hospital, RCSI.

The second approach was the development of peptide prodrugs based on an oligoglutamic acid promoiety. The results of this project show that a short oligoglutamic acid sequence extended by an enzyme-sensitive linker can reversibly mask the activity of the peptide. The HDP peptide alone exhibits activities in the low micromolar range, so it is a viable candidate in its own right, as well as in combination with other anticancer agents. The cathepsin B-sentitive linker was first used as the trigger for the prodrug activation and its eligibility for prodrug generation was indicated through comparison with non proteolytically sensitive linker based negative control, and assessment of respective activities across a number of different cell lines. In collaboration with Dr. Warren Thomas, Beaumont Hospital, RCSI, the development of these peptide prodrug was shown to be promising against mesothelioma cells. Targeting MMP-2 for the activation of the prodrug appears to allow the restoration of the P18-based peptide candidate. The latter displays activities in the low micromolar range across various malignant mesothelioma cell lines and associated with the simplicity in its prodrug design makes it an exciting candidate as a prodrug going forward. Because of the poor clinical record associated with the treatment of malignant mesthelioma, these

prodrugs offer a potential therapy for this condition. The methodology of using these oligoglutamic acid promoieties has also been implemented to generate antimicrobial prodrugs candidates evaluated in collaboration with Dr. Fitzgerald-Hughes.

The third approach was based on a heterodimeric peptide. Conjugation involving peptides sequences can be challenging and associated with scalability limitations for biological testing. Disulfide-based candidates in particular are associated with additional difficulties as they are prone to homodimerization and oxidation. Careful controls during the synthesis of these compounds were necessacry in order to achieve the compound in exploitable yields. In the project a HDP was used both as a vector for a pro-apoptotic peptide and an active anticancer agent. Its assessment was performed in collaboration with Professor Jochen Prehn, Physiology Department, RCSI, and the preliminary results show that the heterodimer peptide is able to induce enhanced cytotoxic effects as compared to its individual components alone and that these effects are occurring through the mitochondrial apoptotic pathway.

The use of peptide prodrugs is a way of combating many of the problems associated with drug delivery. Host defence peptides can also offer a solution in therapeutic areas characterised by an unmet medical need. Simply by changing or altering the peptide sequence, the properties and target of the drug can change. The technology of a developing a prodrug concept to target and deliver different drugs to a wide range of biological targets is becoming a viable approach to combating different types of pathological conditions.

Chapter 7

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7 References

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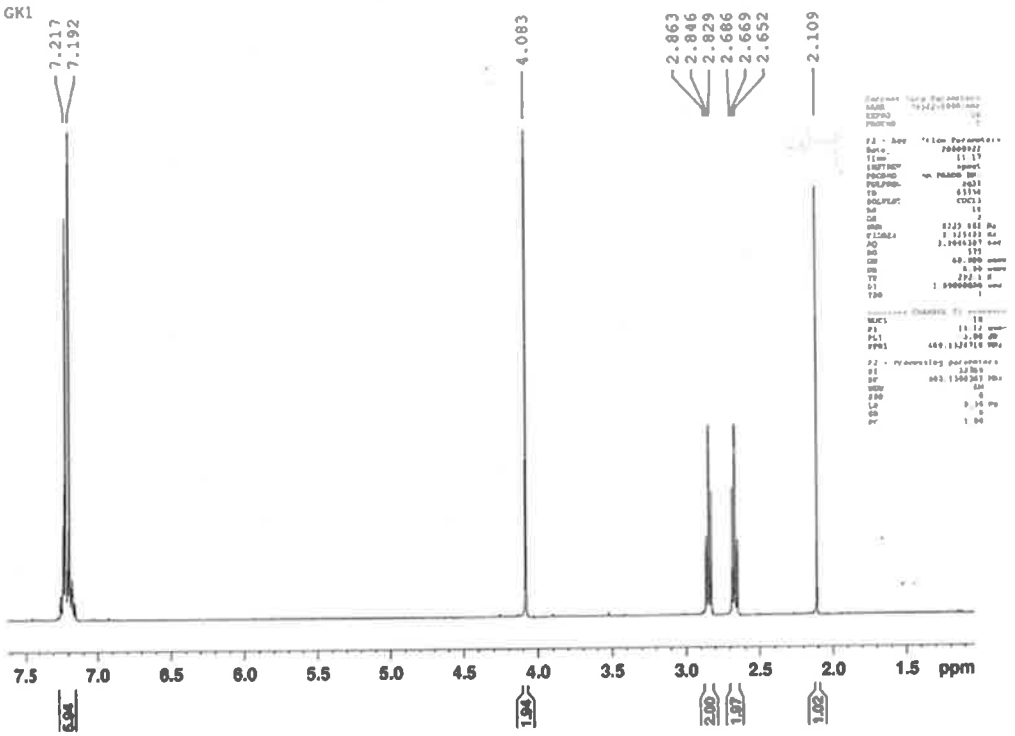
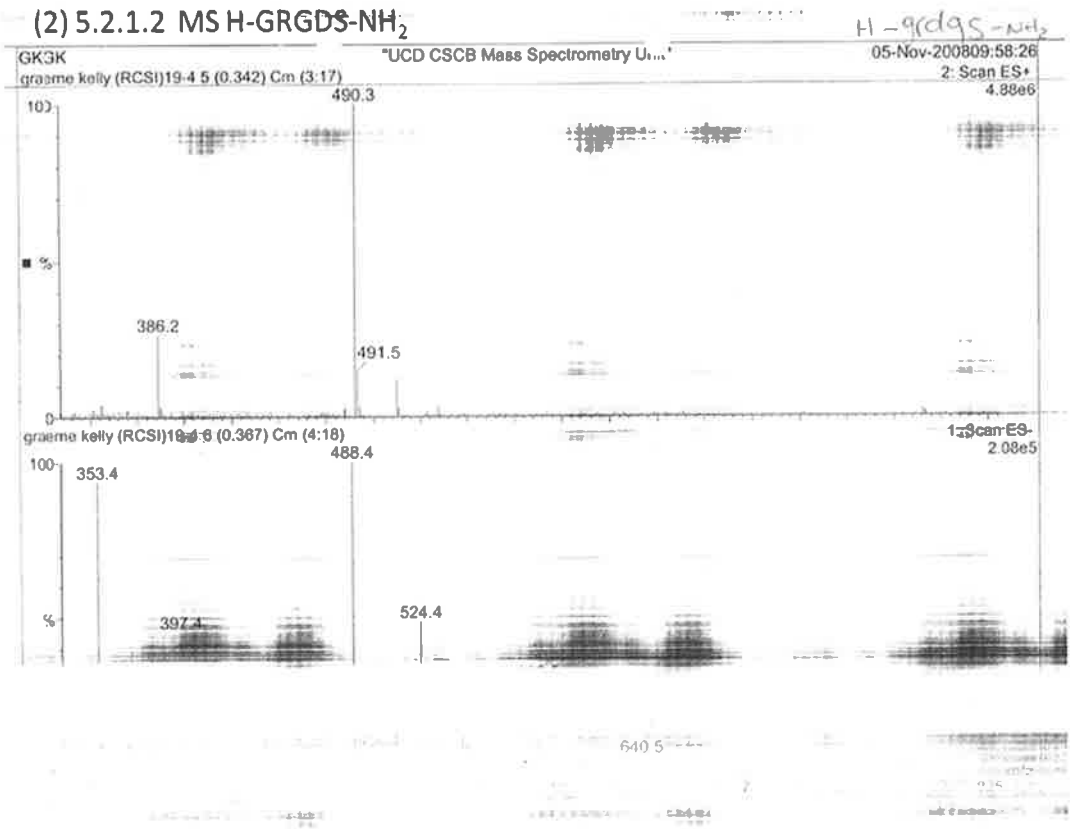
Chapter 8

Appendices

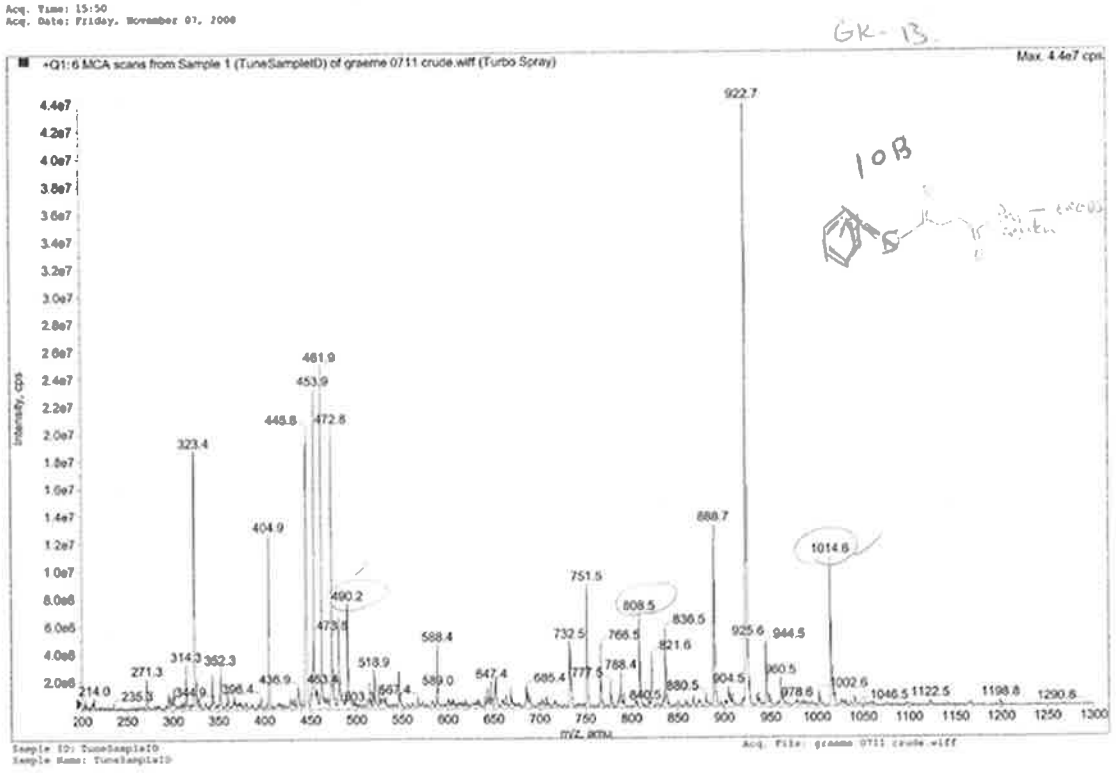
(1) 5.2.1.1 ^{1}H NMR of S-benzyl thiosuccinic acid



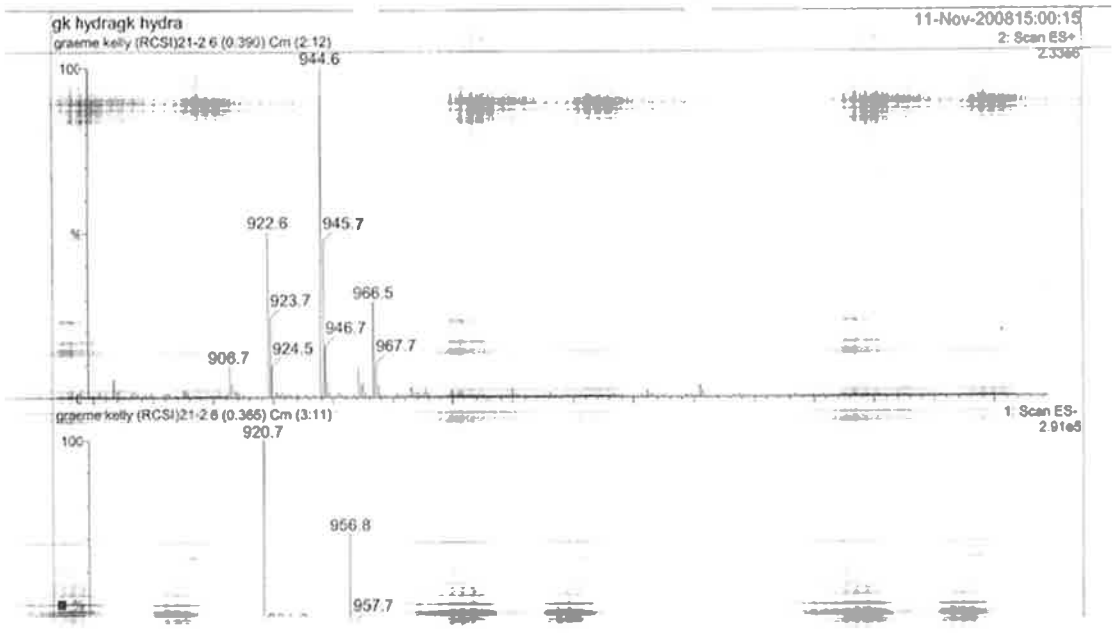
(2) 5.2.1.2 MS H-GRGDS-NH_2



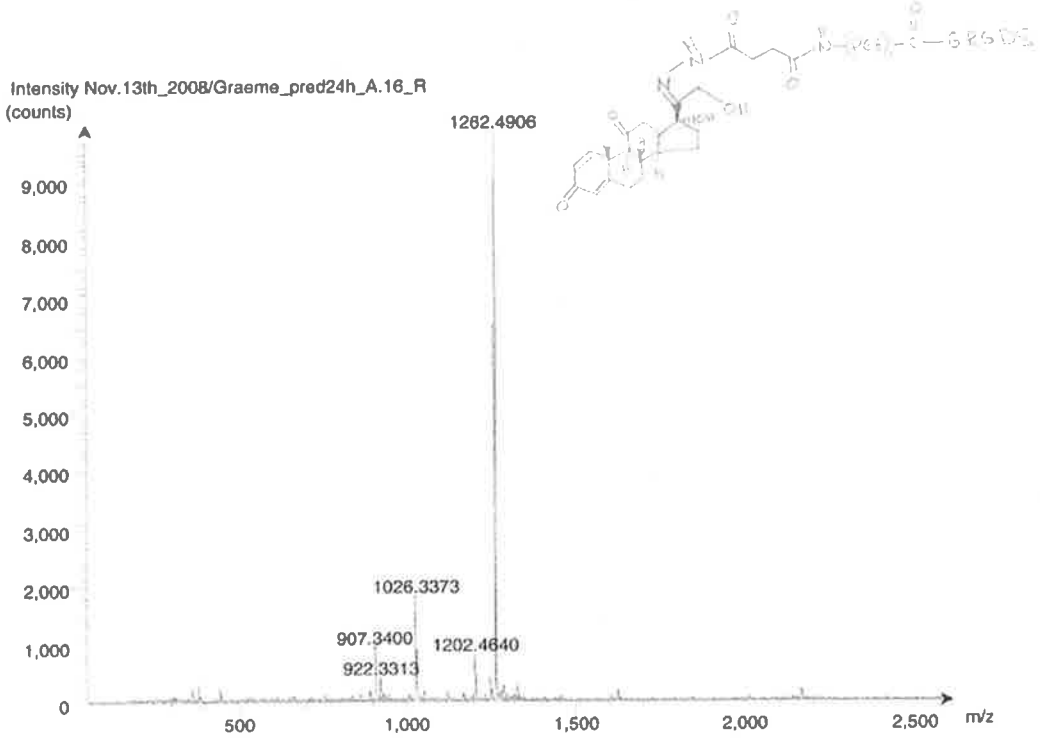
(3) 5.2.1.4 MS S-Benzyl-$C_4O_2H_4$-GRGDS-NH_2



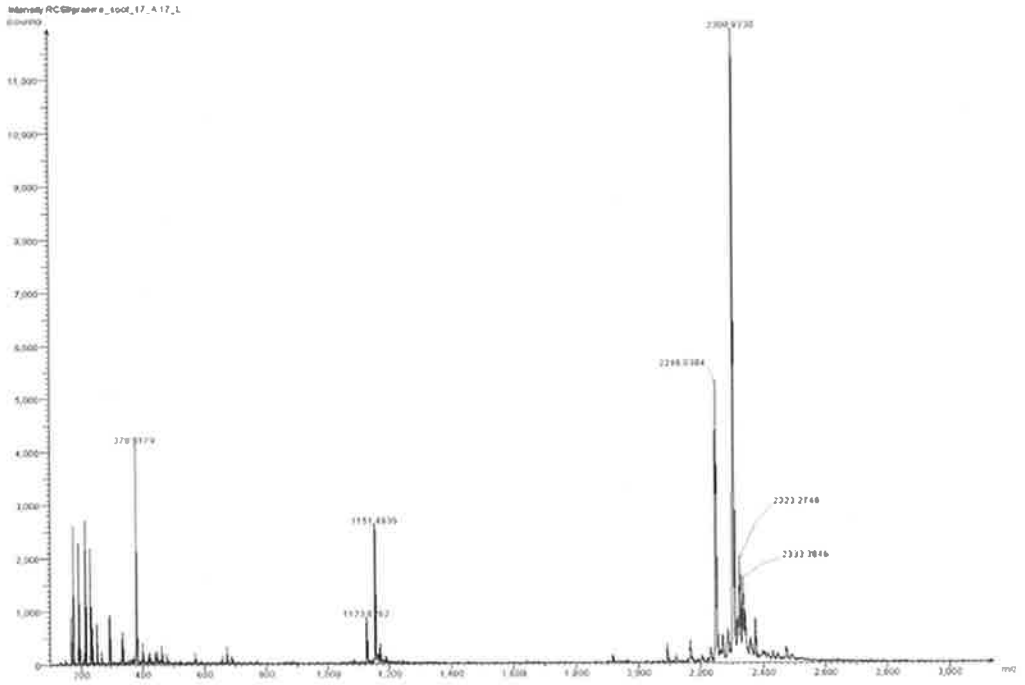
(4) 5.2.1.5 MS H_2N-NH-$C_4O_2H_4$-GRGDS-NH_2



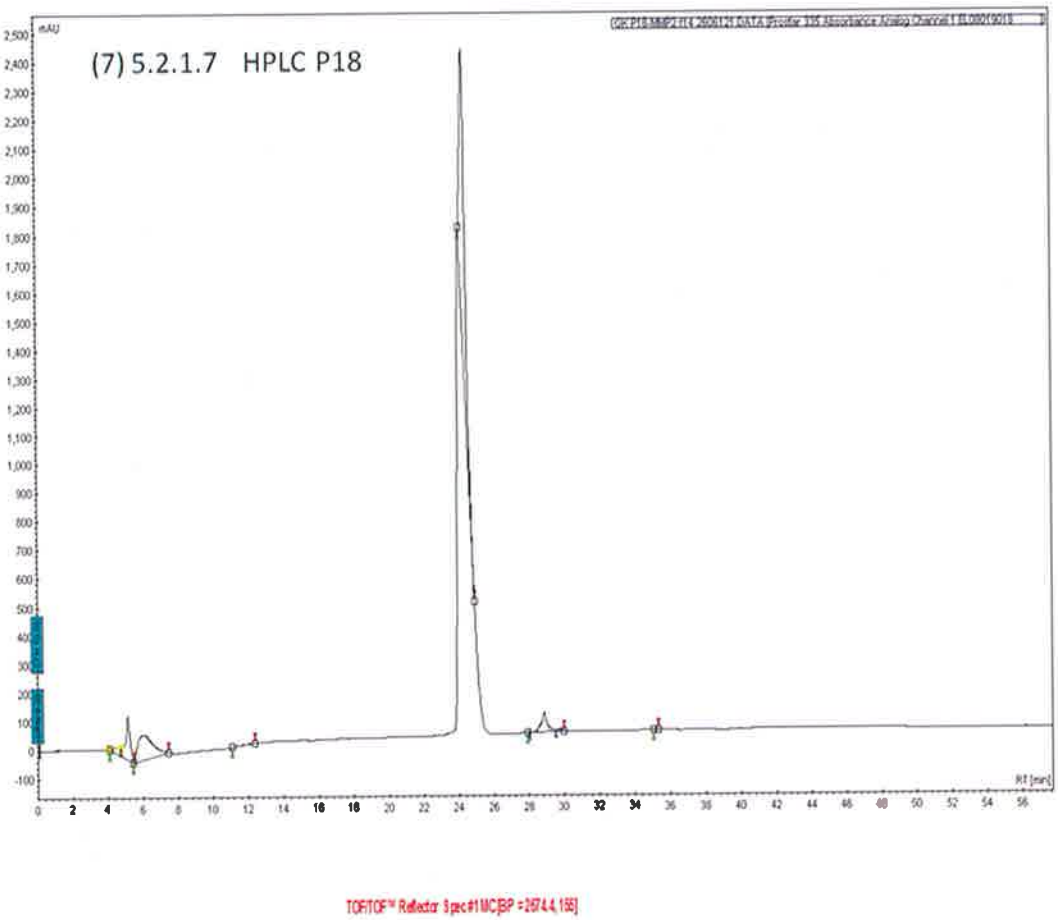
(5) 5.2.1.6 MS Prednisone-N-NH-$C_4O_2H_4$-GRGDS-NH_2



(6) 5.2.1.7 MS P18

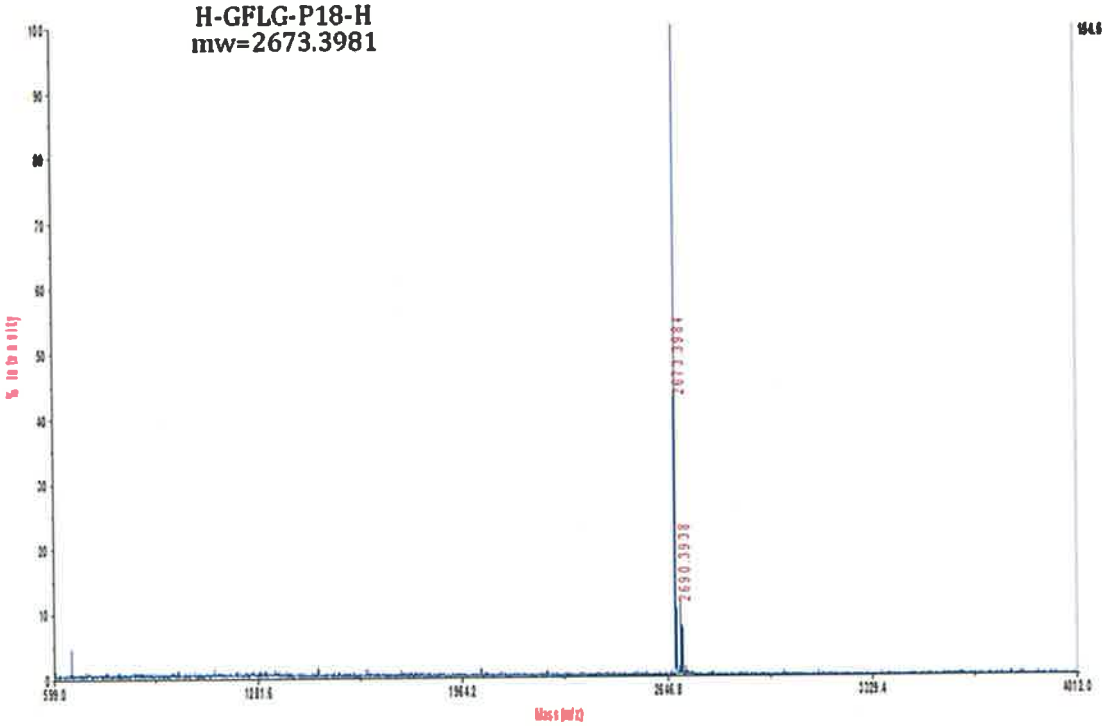


(7) 5.2.1.7 HPLC P18

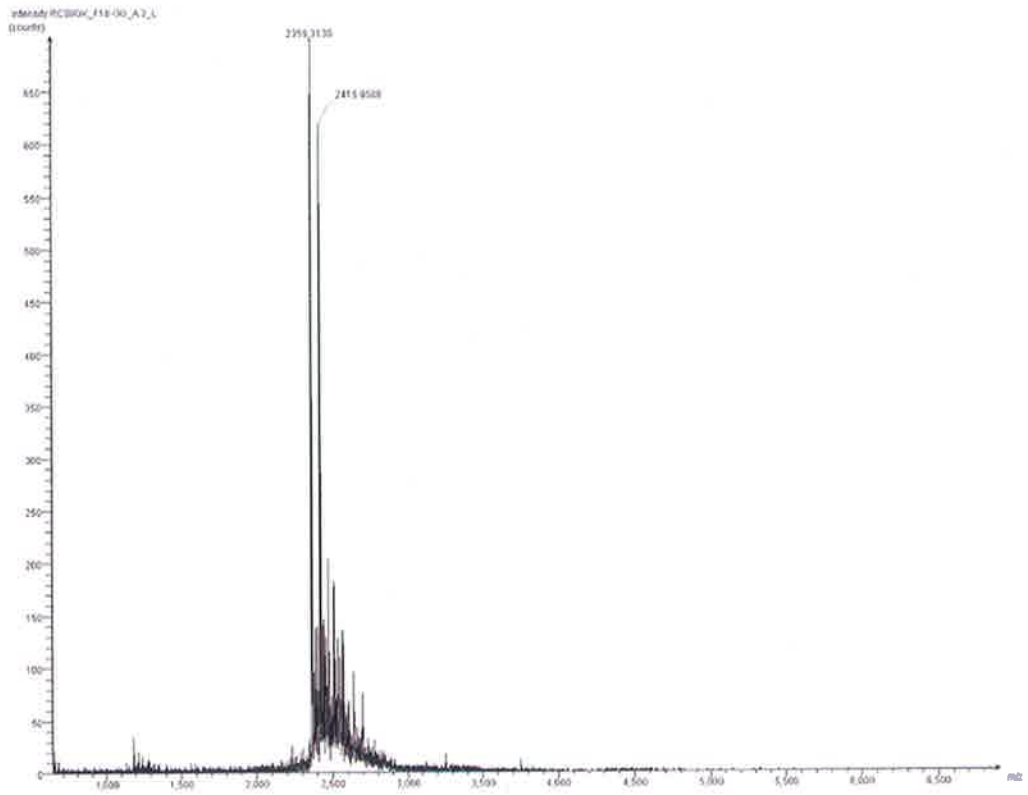


(8) 5.2.1.8 MS H-GFLG-P18-NH_2

H-GFLG-P18-H
mw=2673.3981

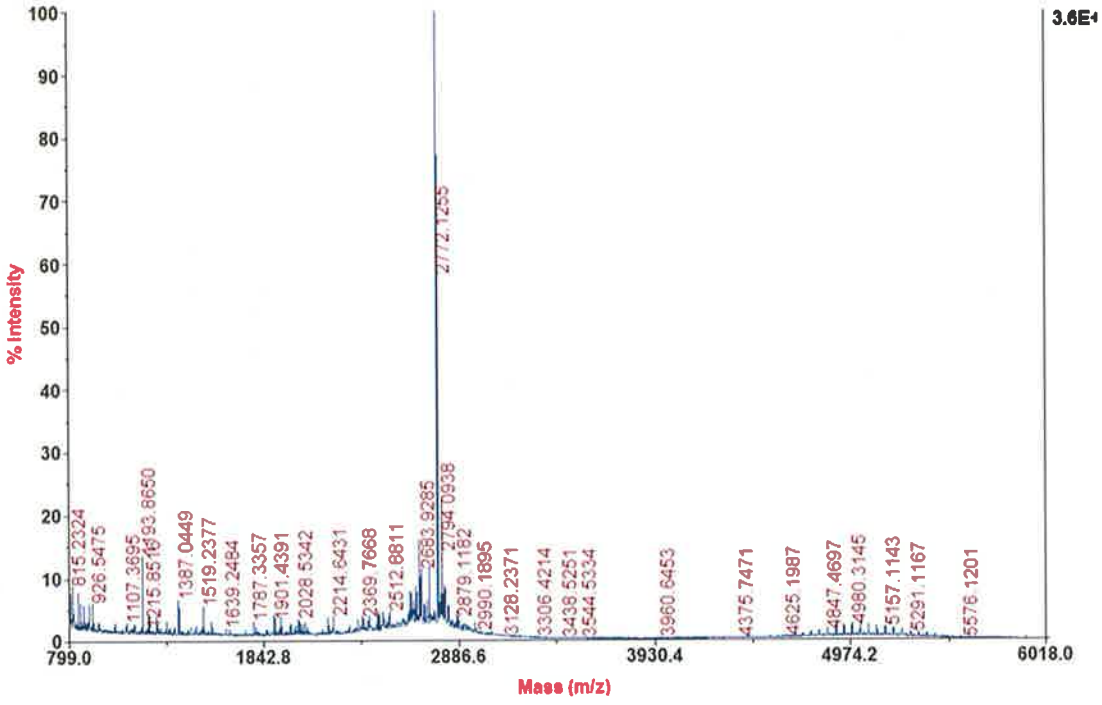


(9) 5.2.1.9 MS H-GG-P18-NH_2

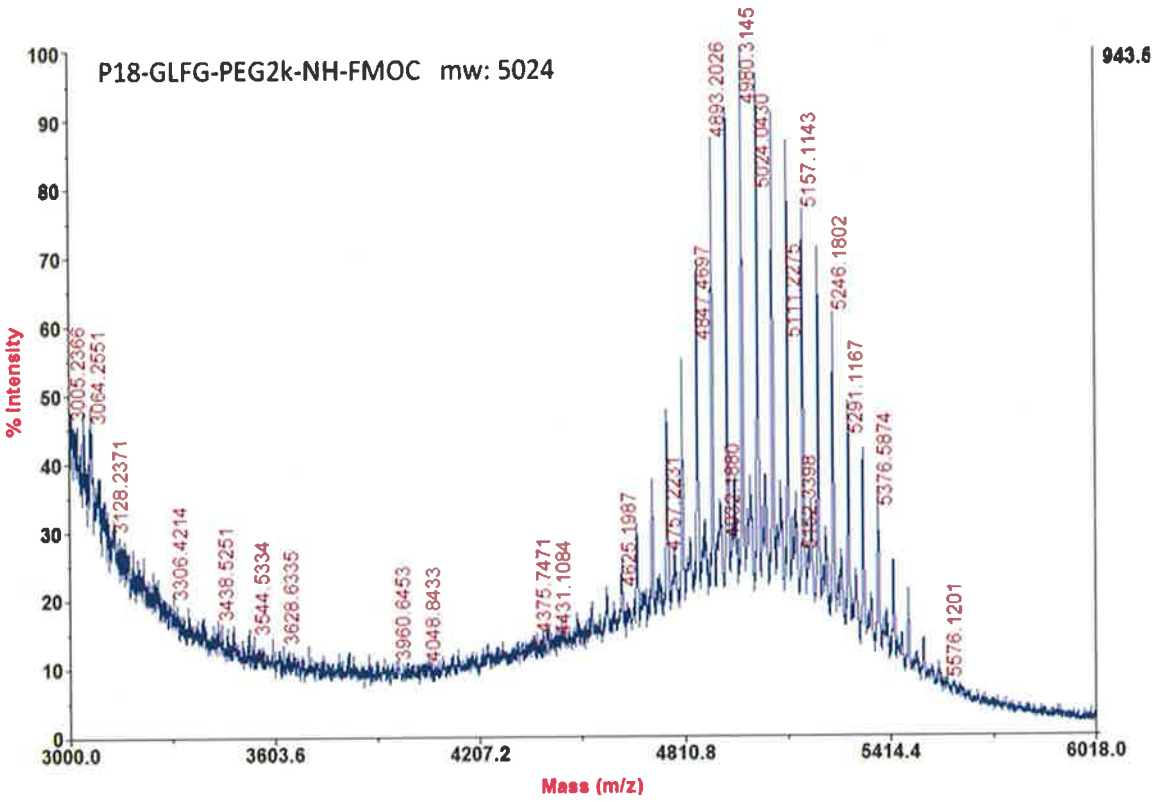


(10) 5.2.1.10

P18-GLFG-PEG2k-NH-FMOC mw: 5024

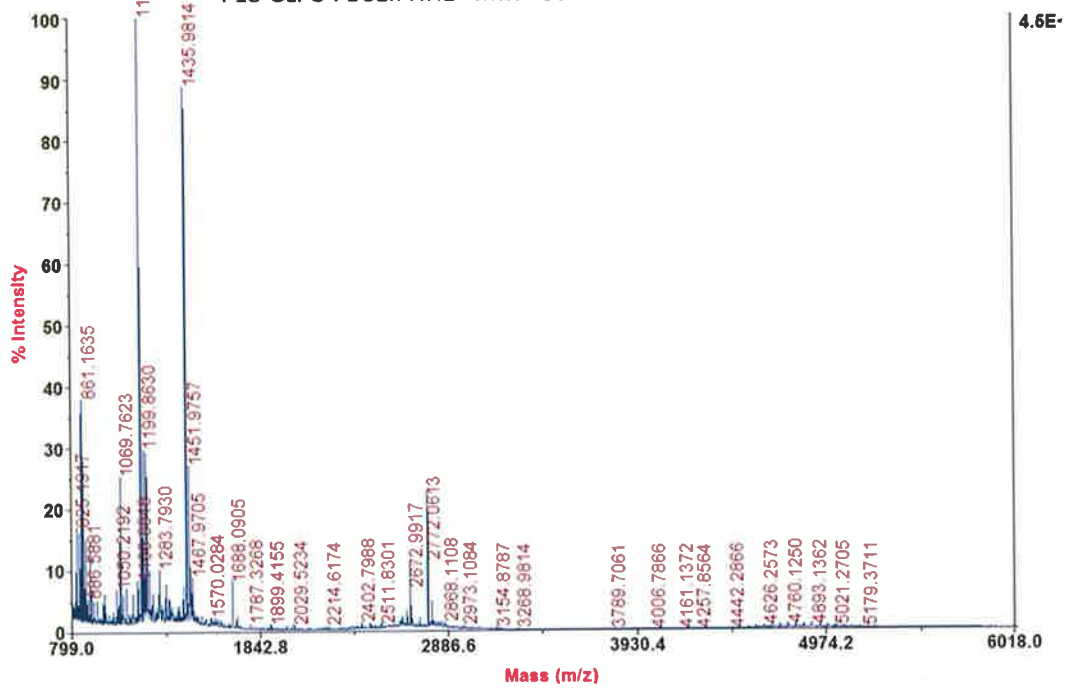


(10A) 5.2.1.10 zoom



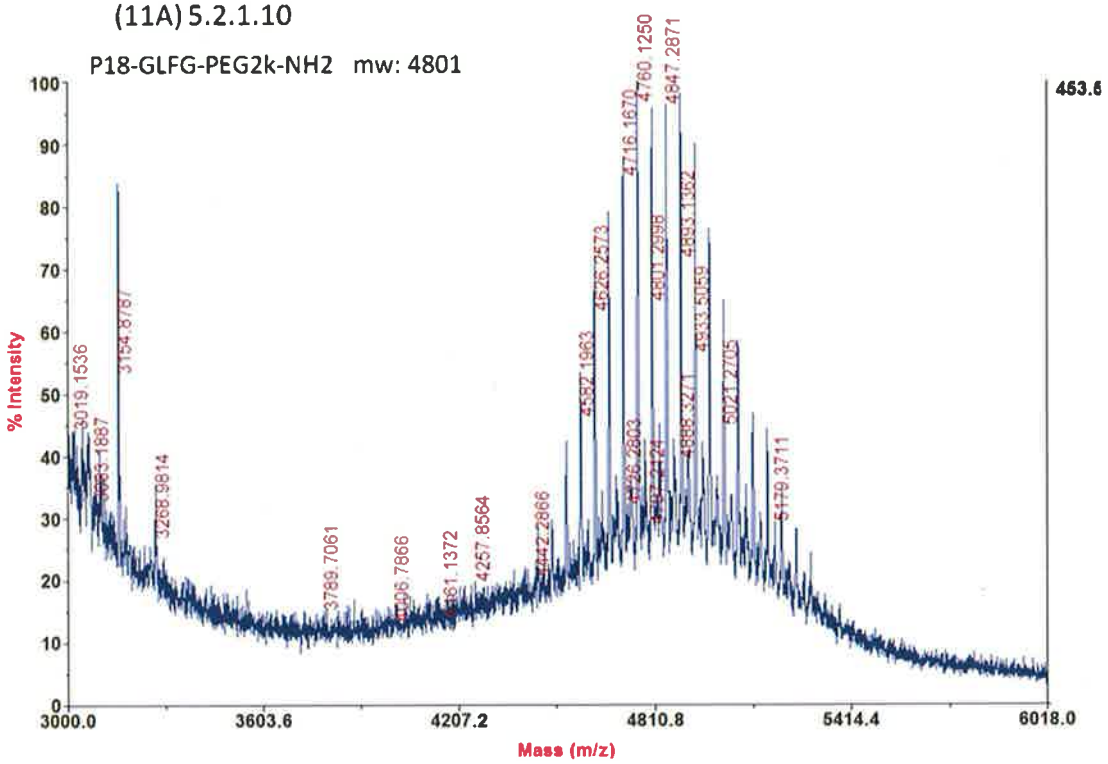
(11) 5.2.1.10

P18-GLFG-PEG2k-NH2 mw: 4801

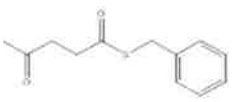


(11A) 5.2.1.10

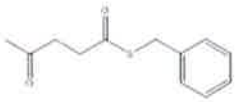
P18-GLFG-PEG2k-NH2 mw: 4801

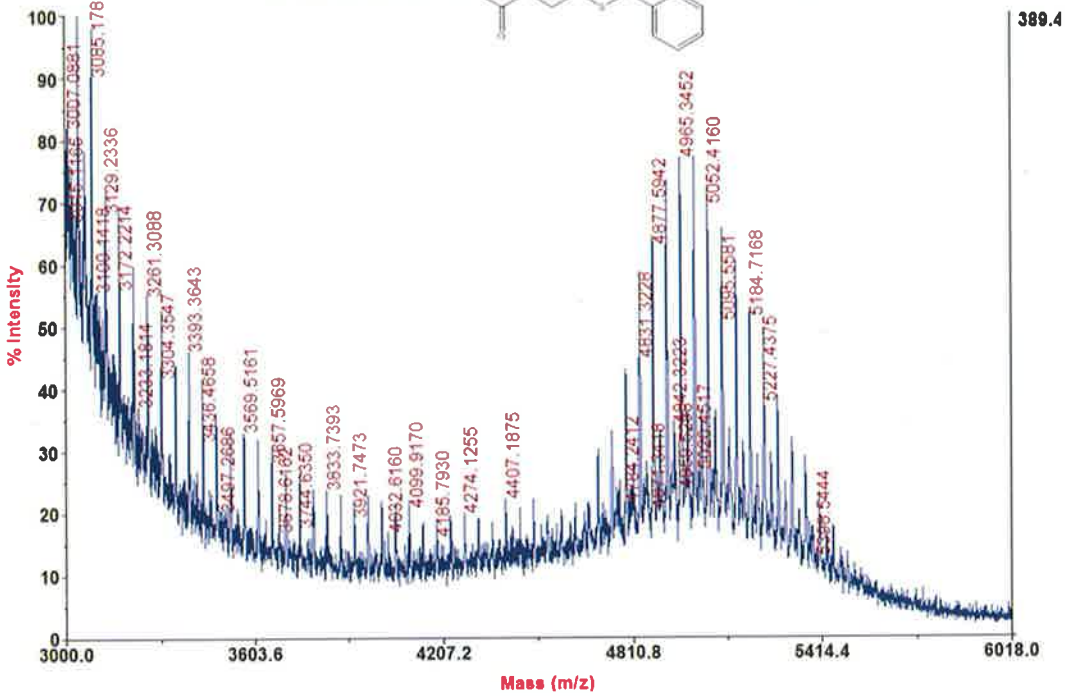


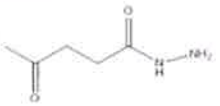
(12) 5.2.1.10

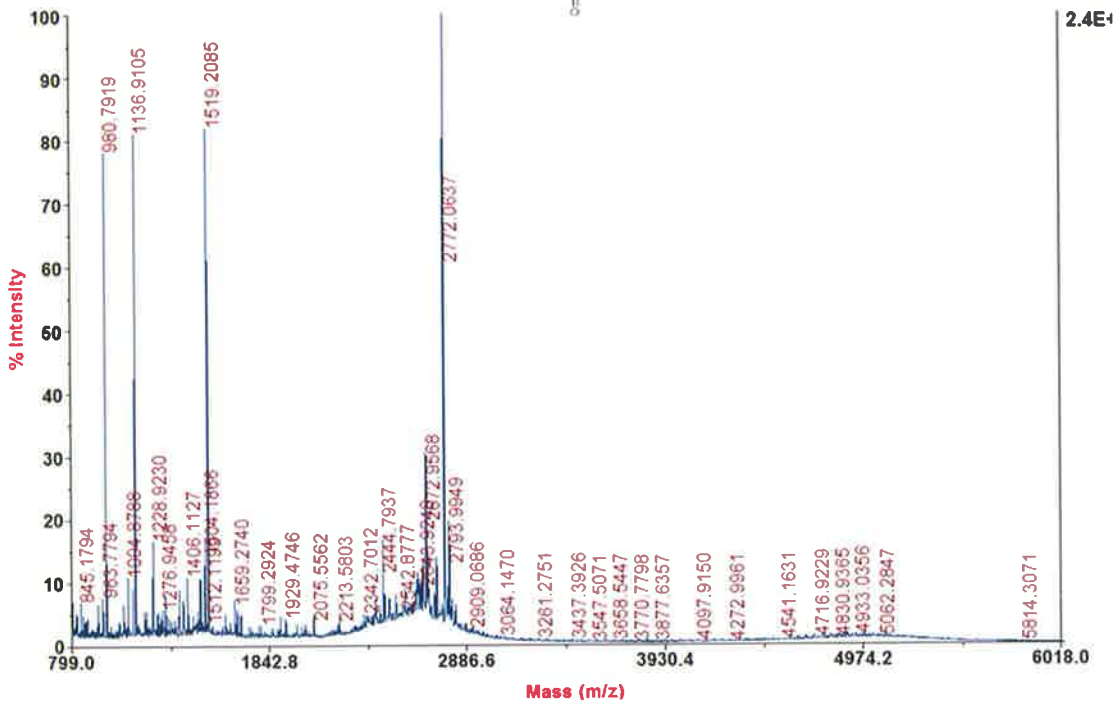
P18-GLFG-PEG2k-NH  mw: 5023

(12A) 5.2.1.10 zoom

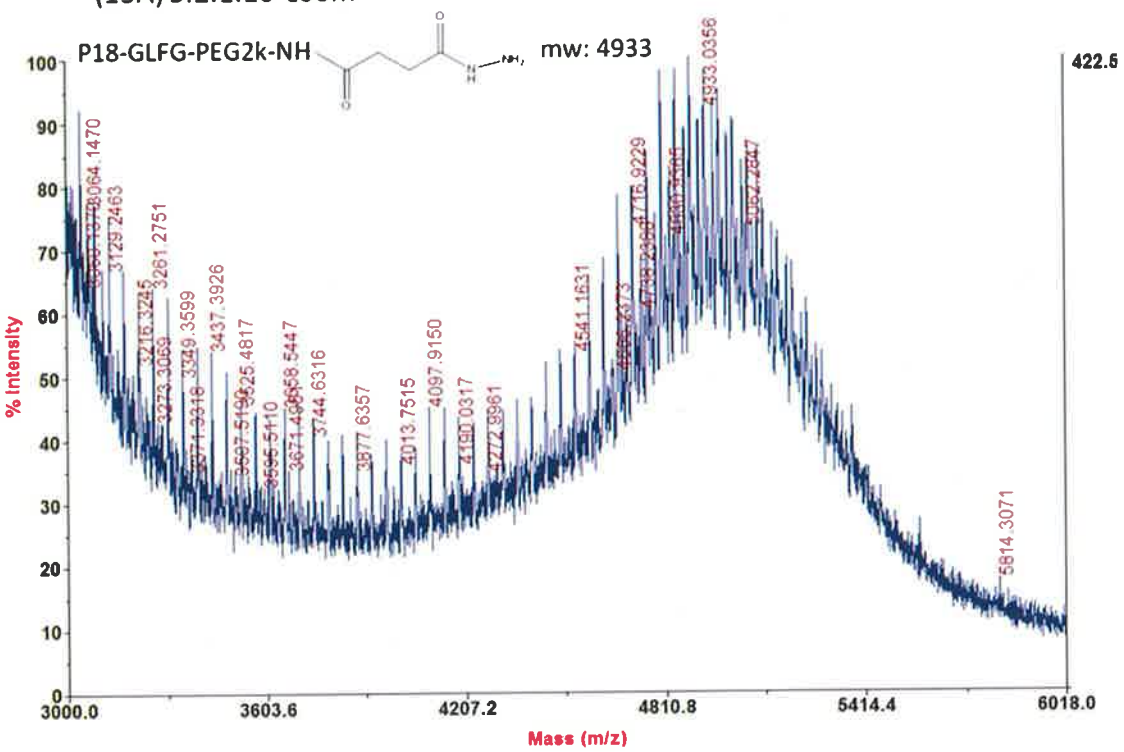
P18-GLFG-PEG2k-NH  mw: 5023



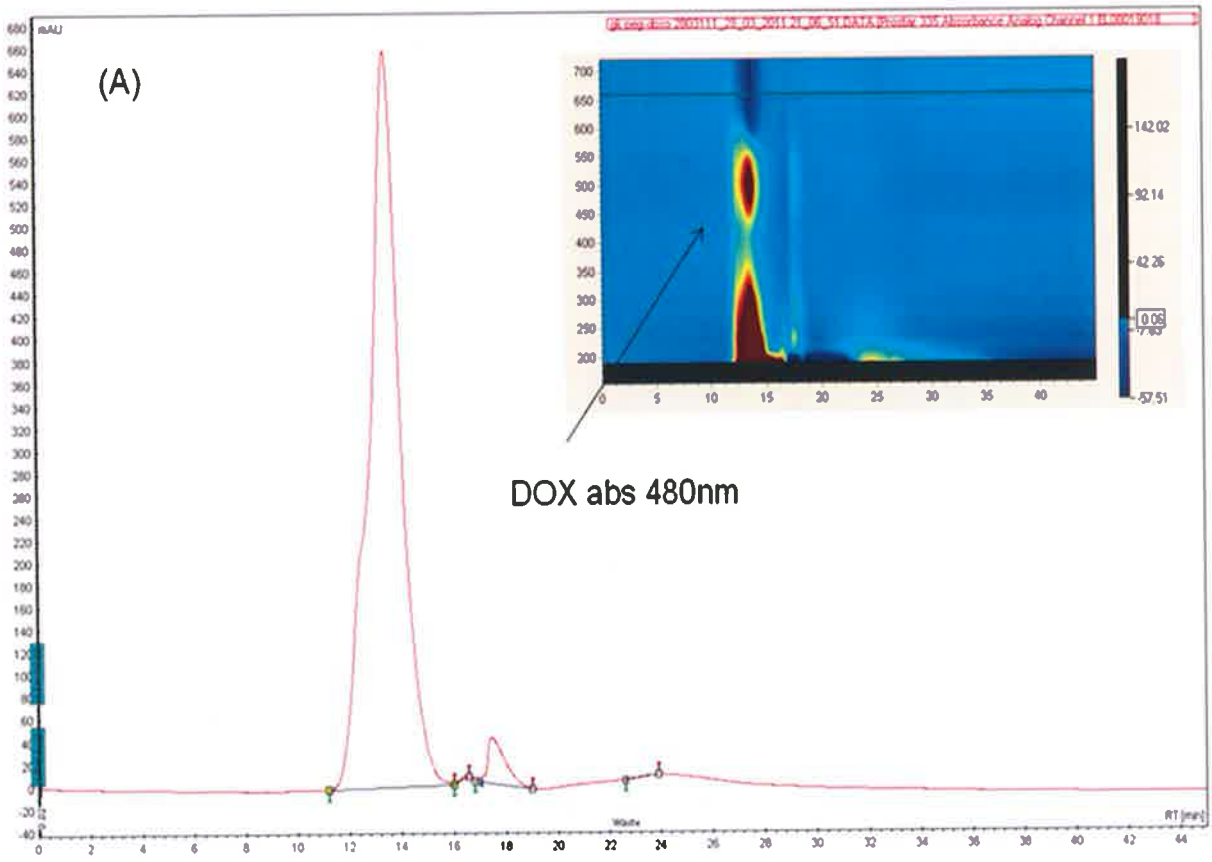
(13) 5.2.1.10 P18-GLFG-PEG2k-NH  mw: 4933



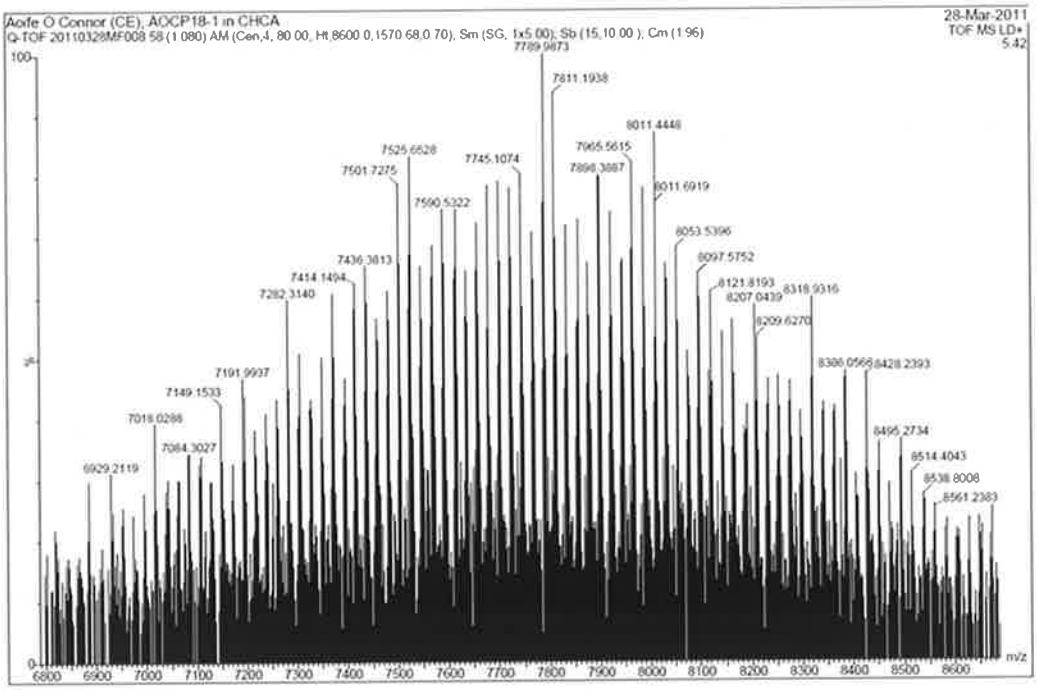
(13A) 5.2.1.10 zoom



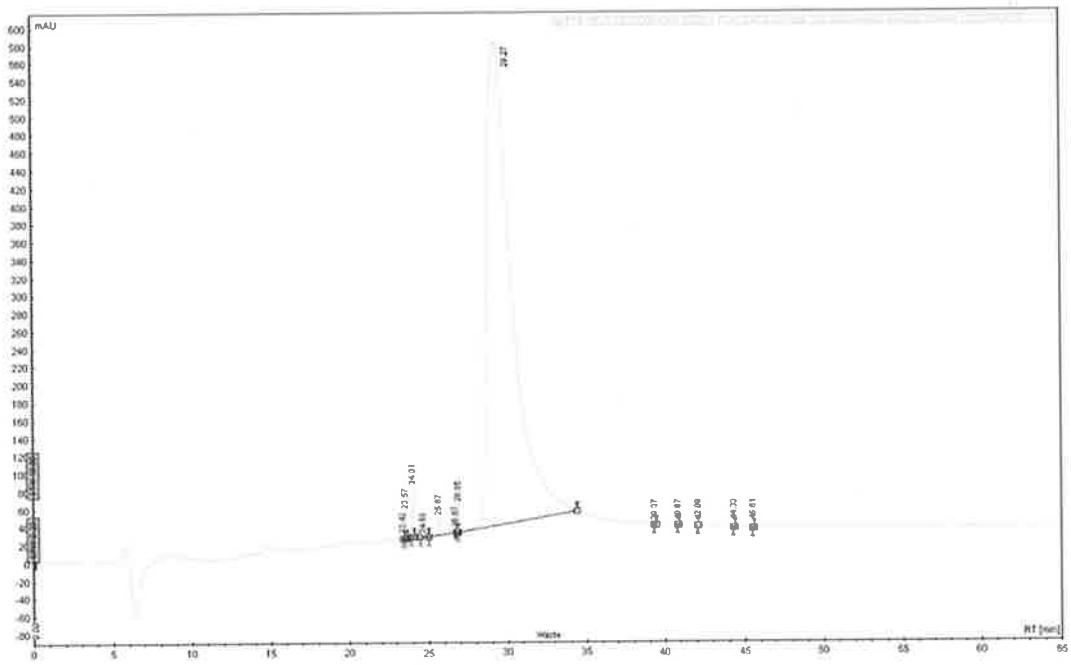
(14) 5.2.1.10 pure dox-peg-pep



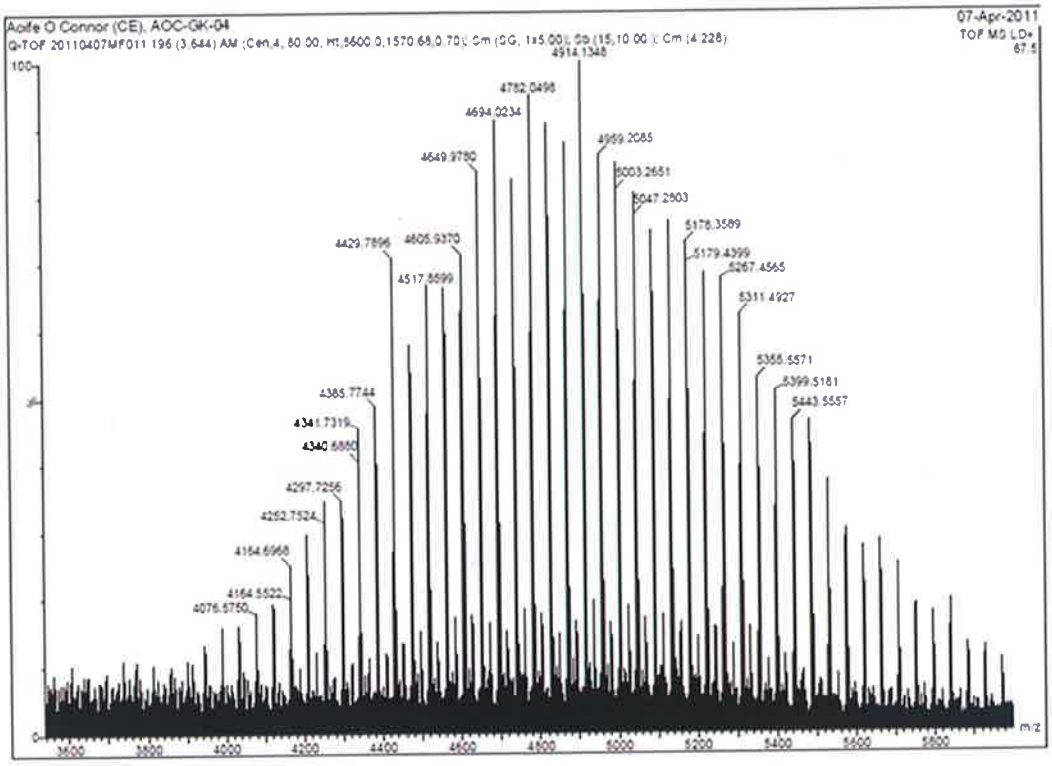
(15) 5.2.1.11 MS MeO-PEG$_{5000}$-GFLG-P18-NH$_2$



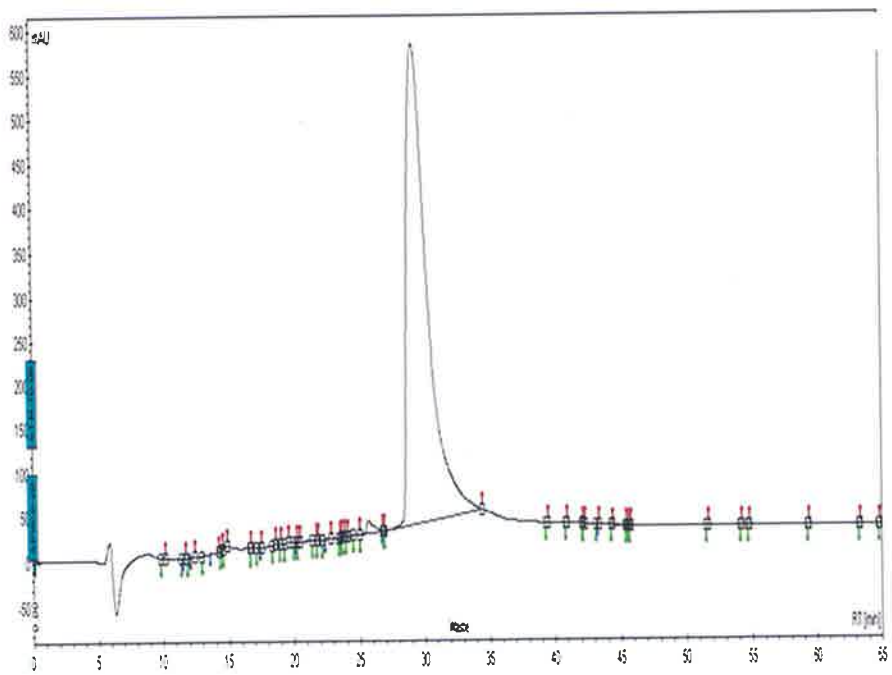
(16) 5.2.1.11 HPLC MeO-PEG$_{5000}$-GFLG-P18-NH$_2$



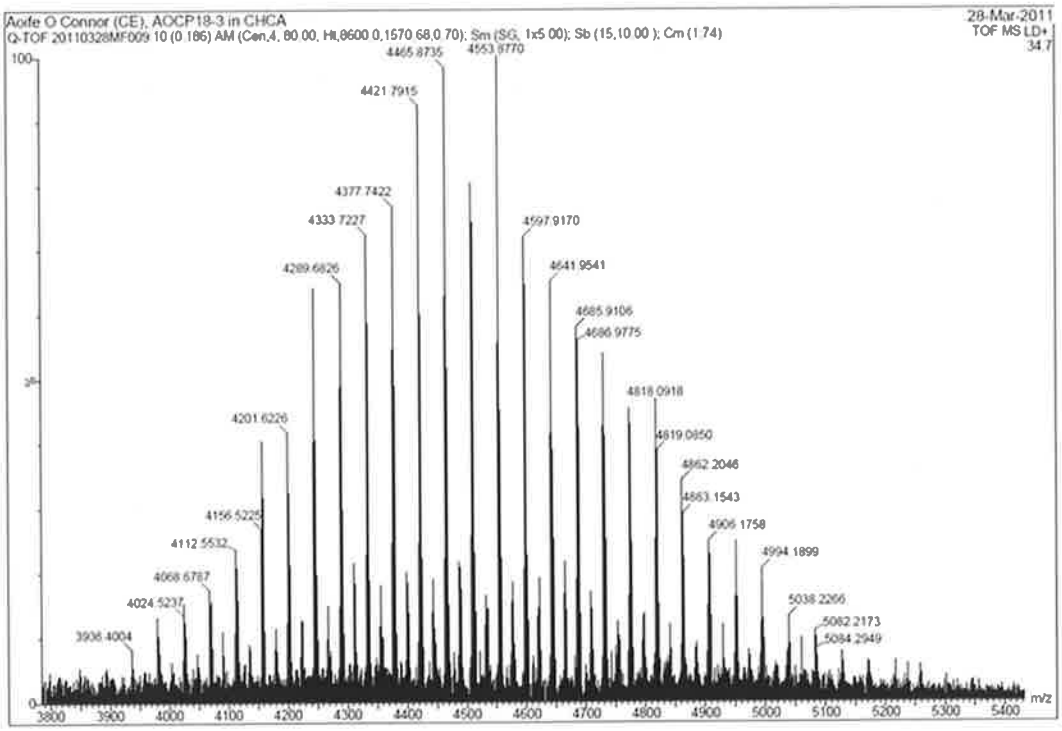
(17) 5.2.1.12 MS MeO-PEG_{2000}-GFLG-P18-NH_2



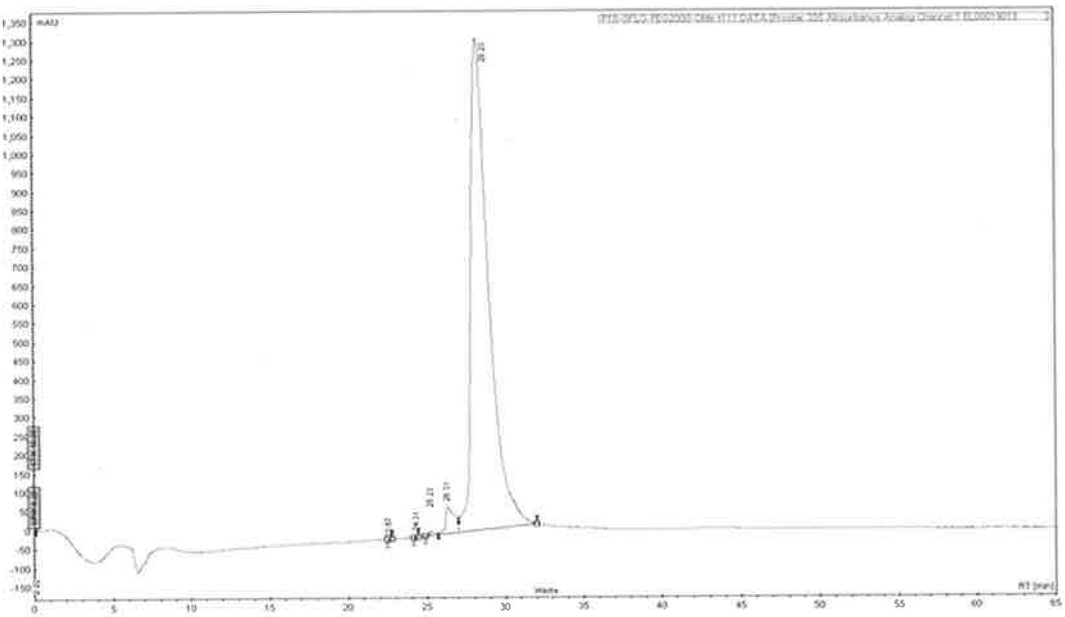
(18) 5.2.1.12 HPLC MeO-PEG_{2000}-GFLG-P18-NH_2



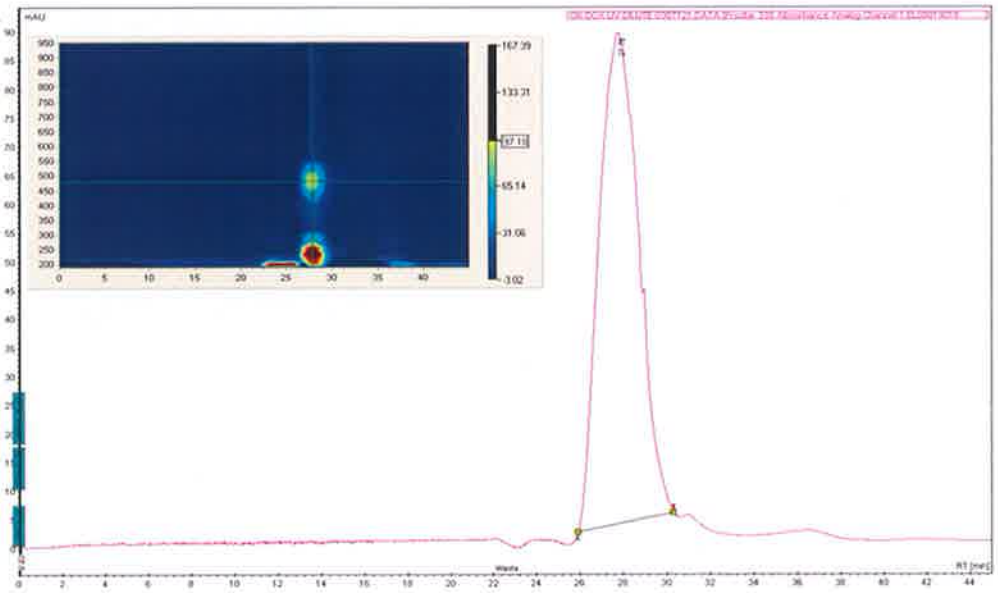
(19) 5.2.1.13 MS MeO-PEG$_{2000}$-GG-P18-NH$_2$



(20) 5.2.1.13 HPLC MeO-PEG$_{2000}$-GG-P18-NH$_2$

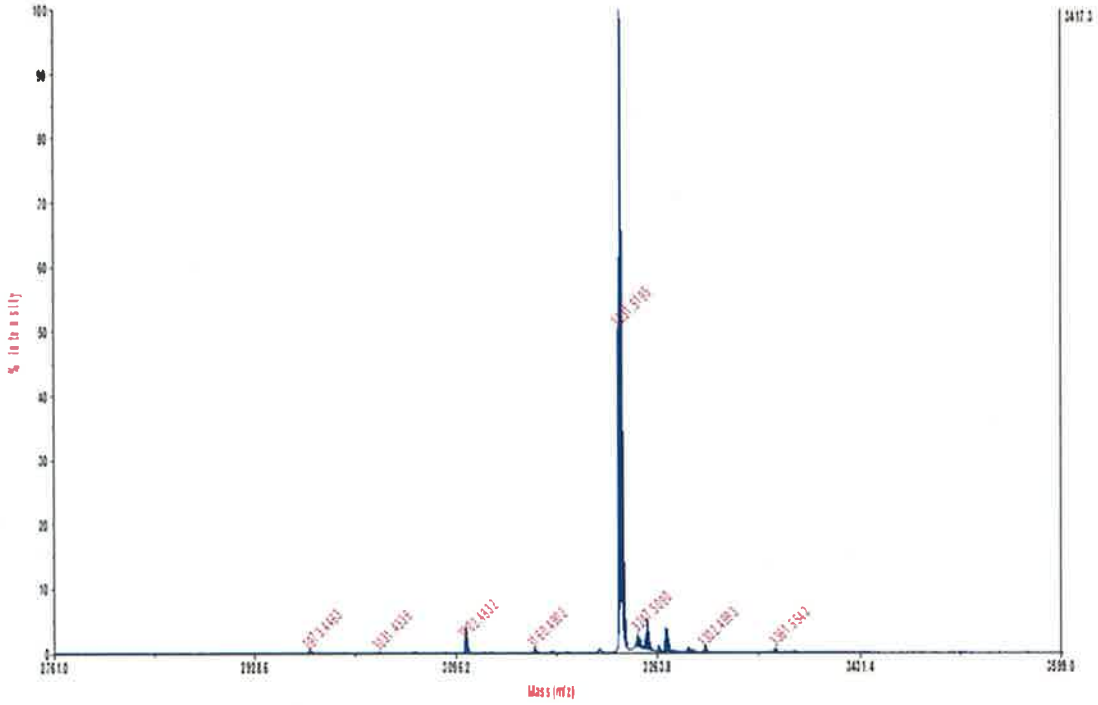


(21) 5.2.1.14 UV-VIS DOX

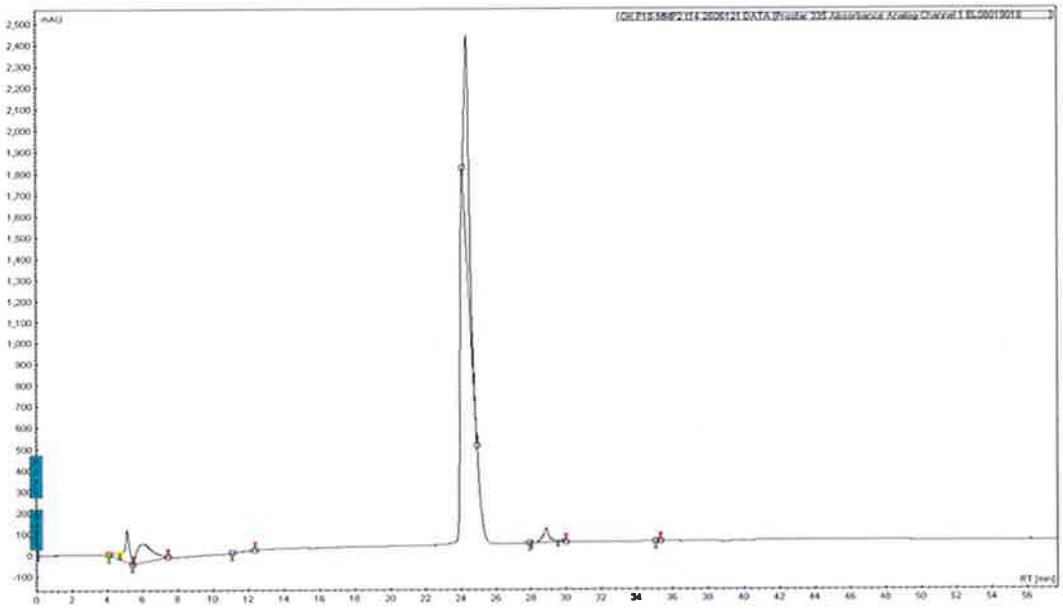


TOF/TOF™ Reflector Spec #1 MC[BP = 3232.5, 3417]

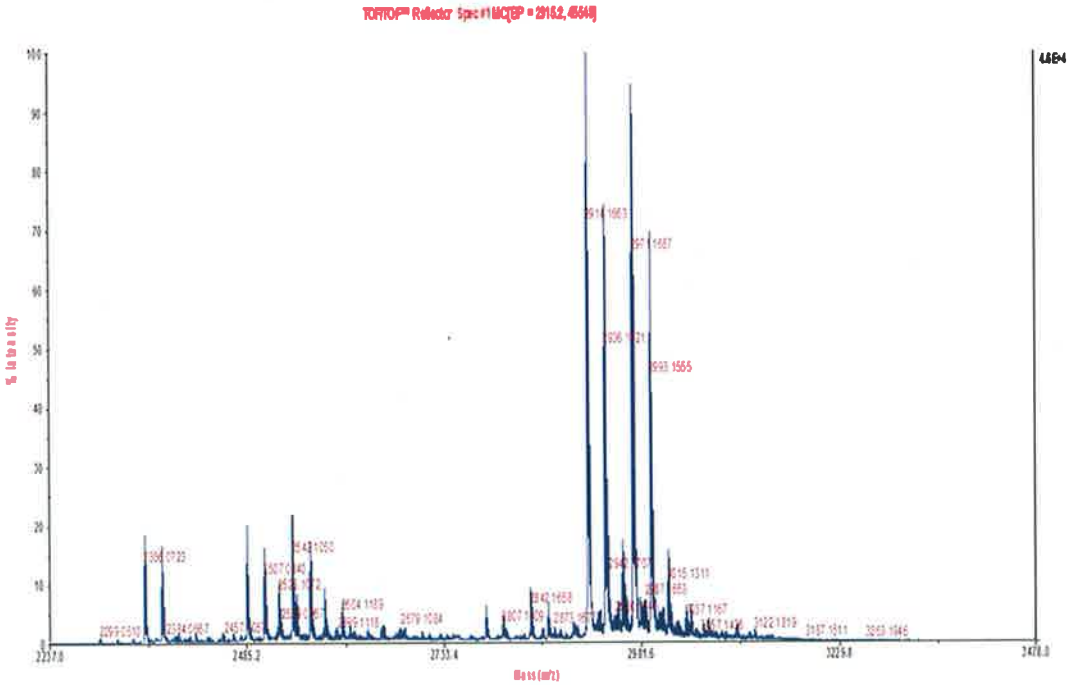
(22) 5.4.1.1 MS Ac-EEEE-GFLG-P18-NH_2



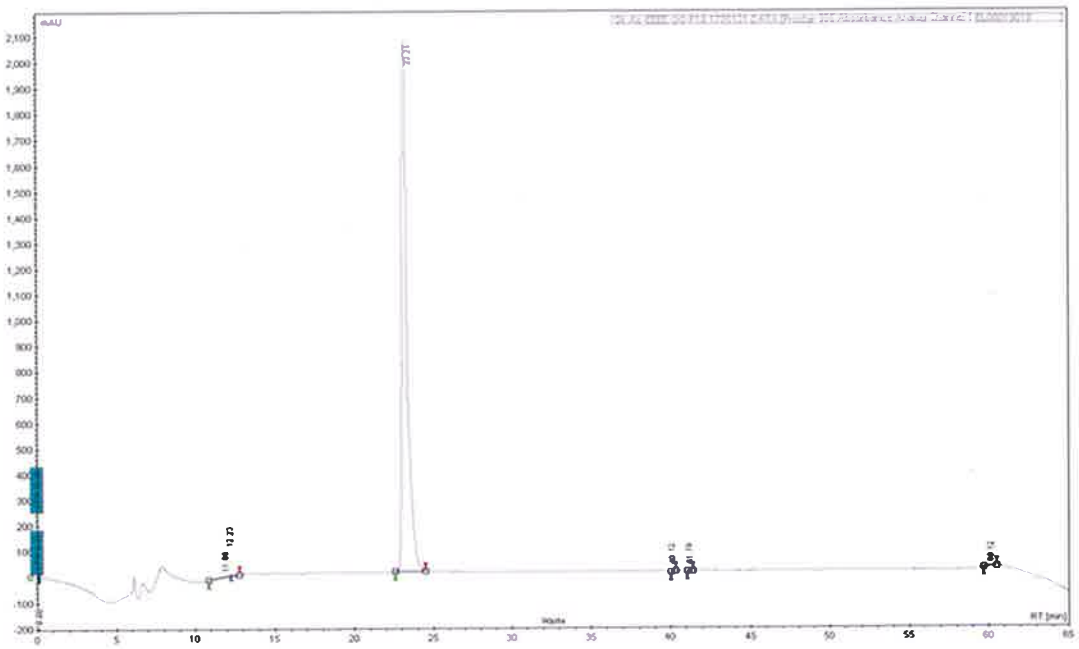
(23) 5.4.1.1 HPLC Ac-EEEE-GFLG-P18-NH_2



(24) 5.4.1.2 MS Ac-EEEE-GG-P18-NH_2

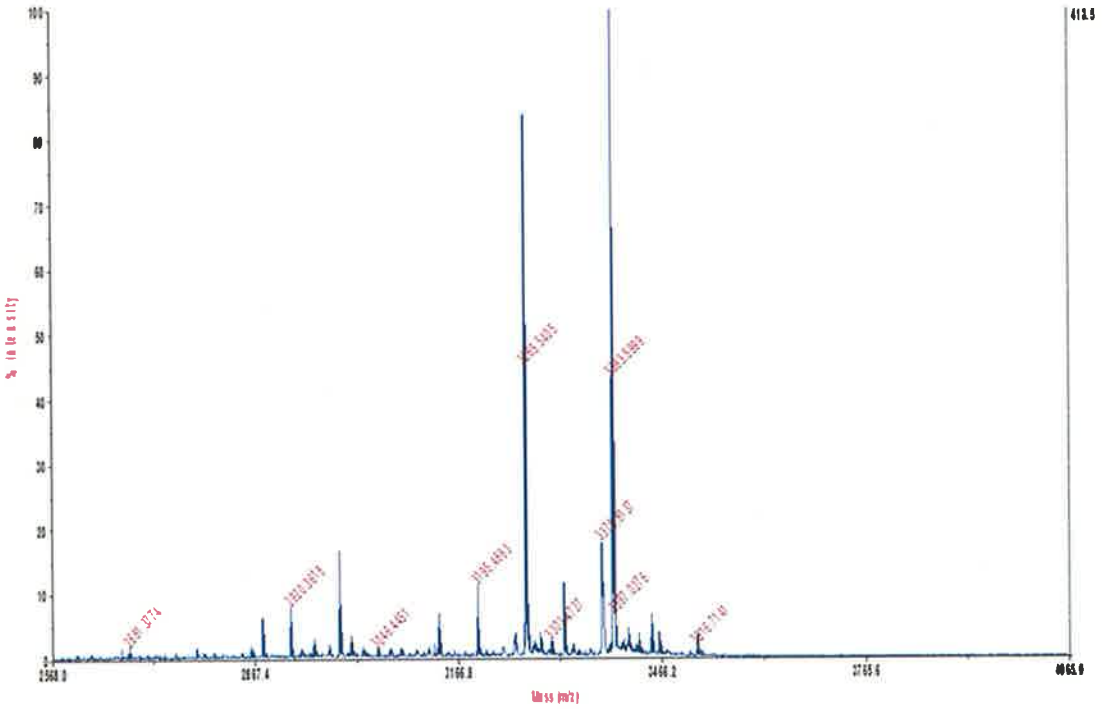


(25) 5.4.1.2 HPLC Ac-EEEE-GG-P18-NH_2

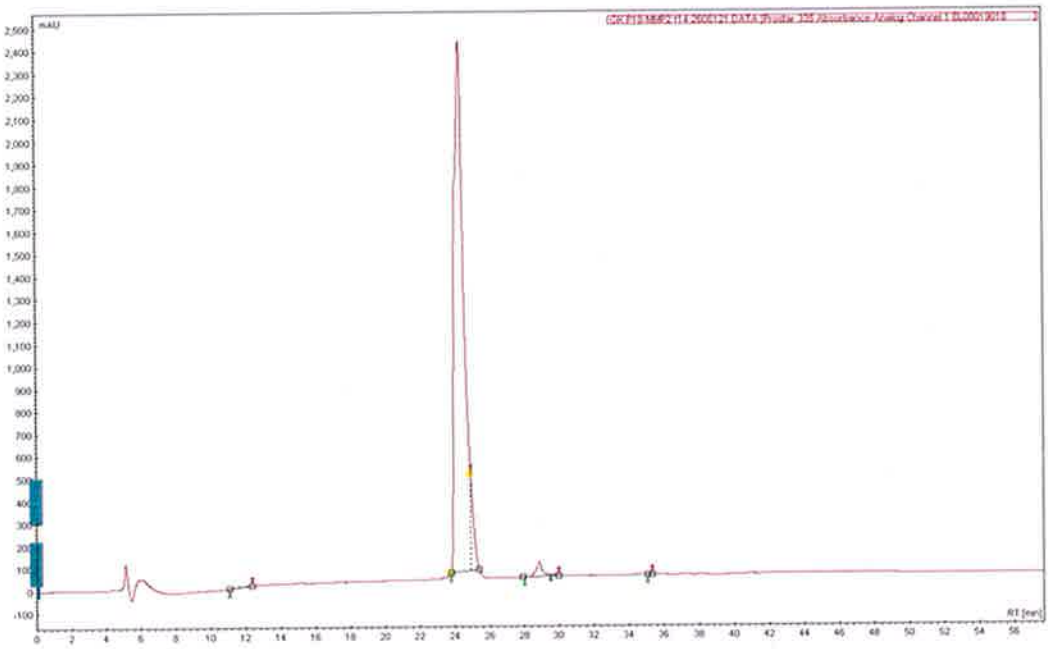


TOF/TOF™ Reflector Spec #1 MC[BP = 2396.6, 413]

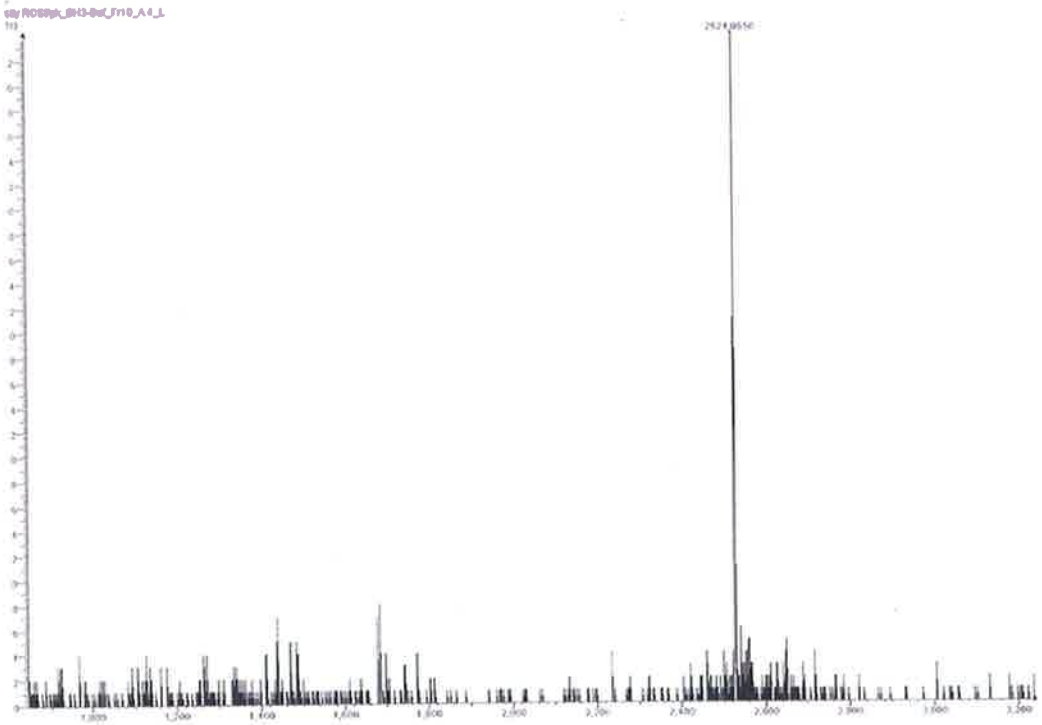
(26) 5.4.1.3 MS Ac-EEEE-PVGLIG-P18-NH_2



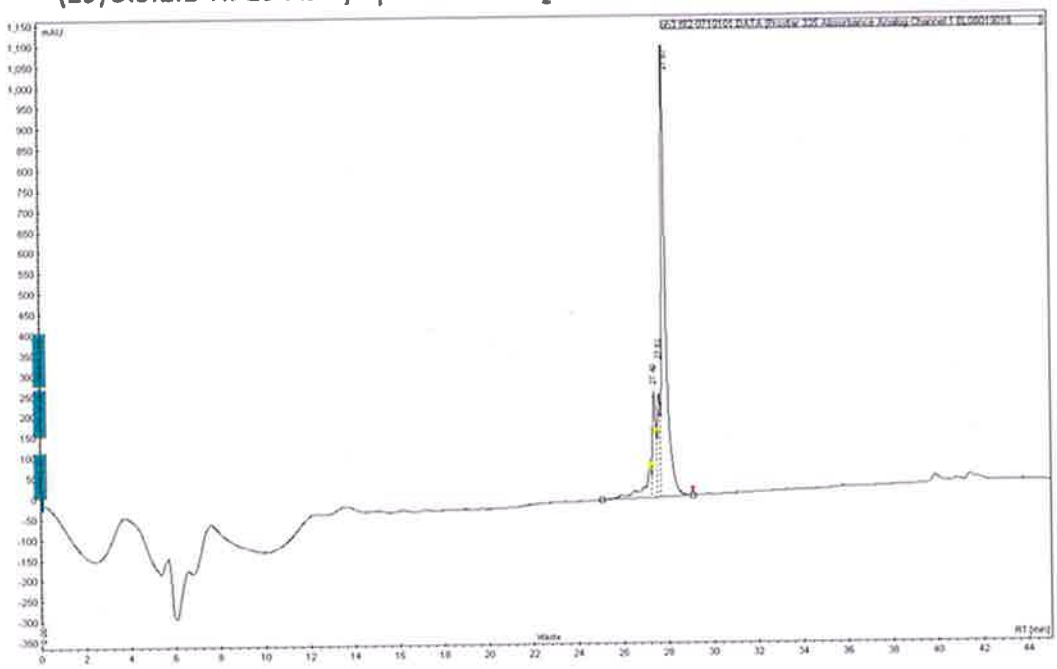
(27) 5.4.1.3 HPLC Ac-EEEE-PVGLIG-P18-NH_2



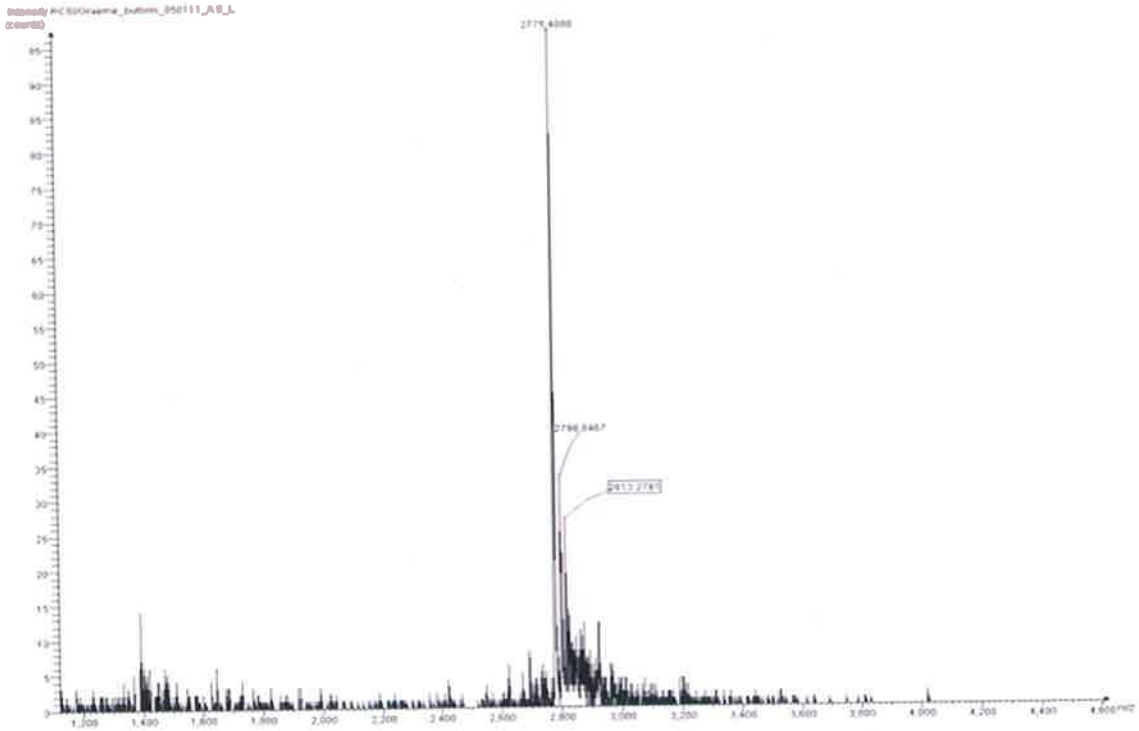
(28) 5.5.1.1 MS Ac-Cys-βAla-BH3-NH_2



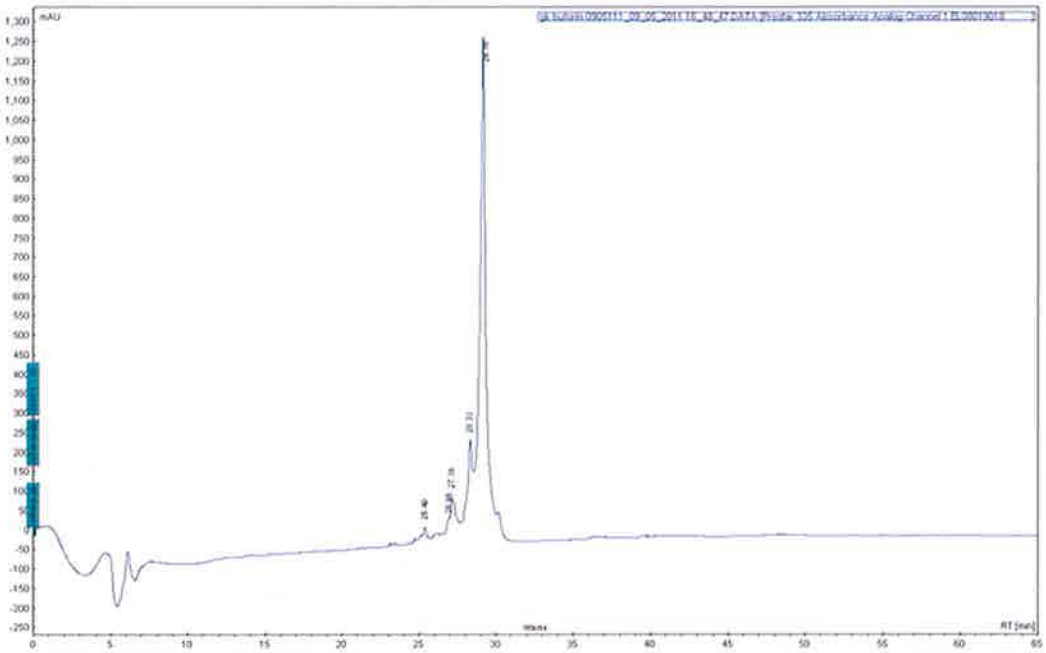
(29) 5.5.1.1 HPLC Ac-Cys-βAla-BH3-NH_2



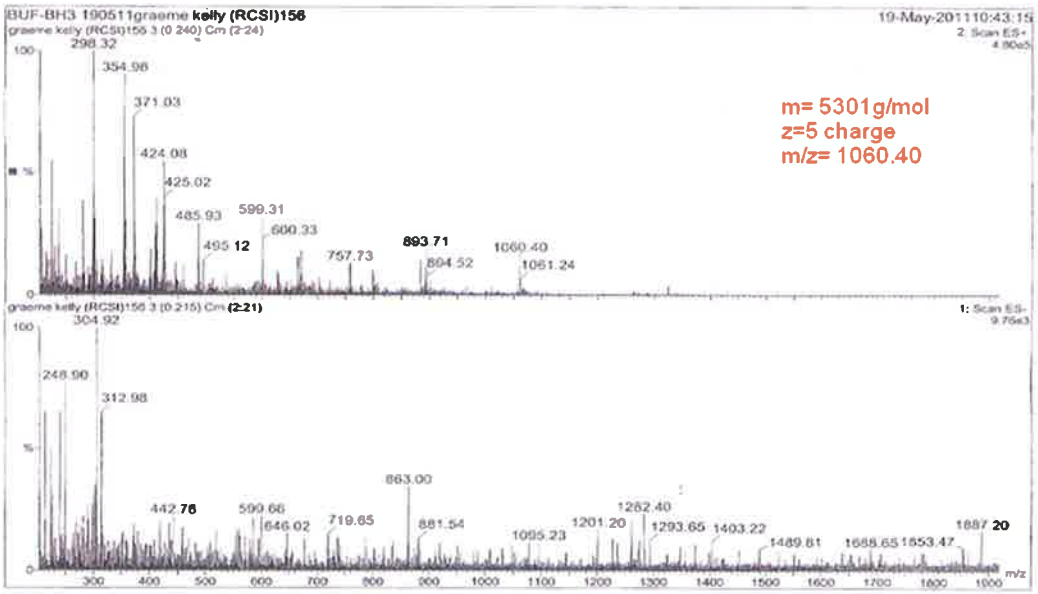
(30) 5.5.1.3 MS Ac-Cys-βAla-Buforin-NH_2



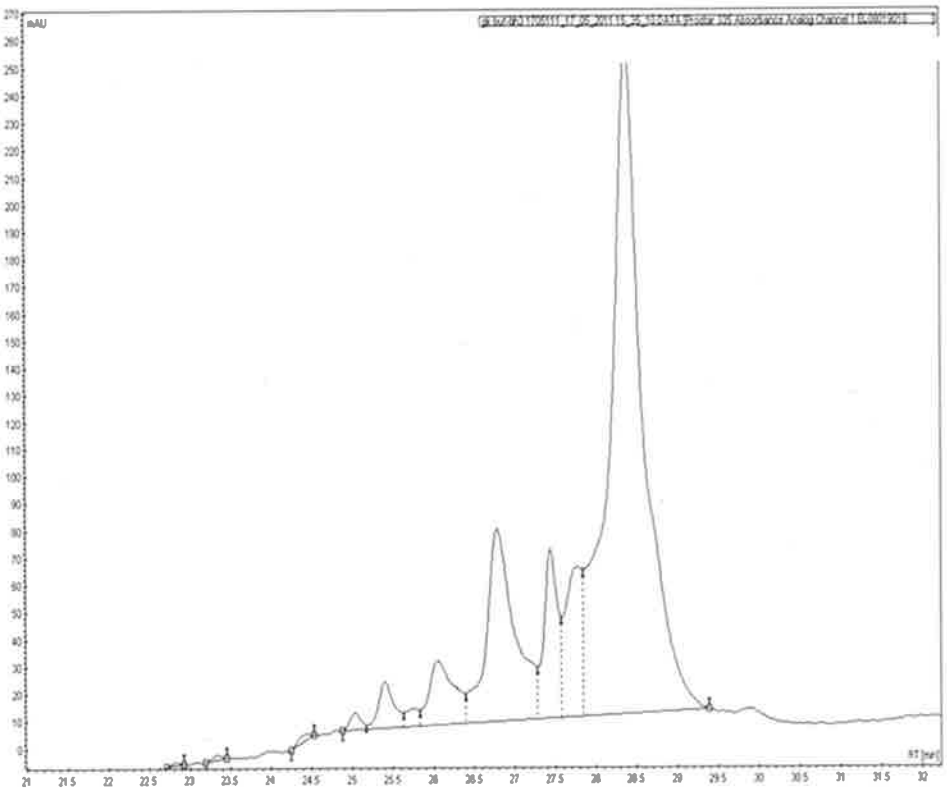
(31) 5.5.1.3 HPLC Ac-Cys-βAla-Buforin-NH_2



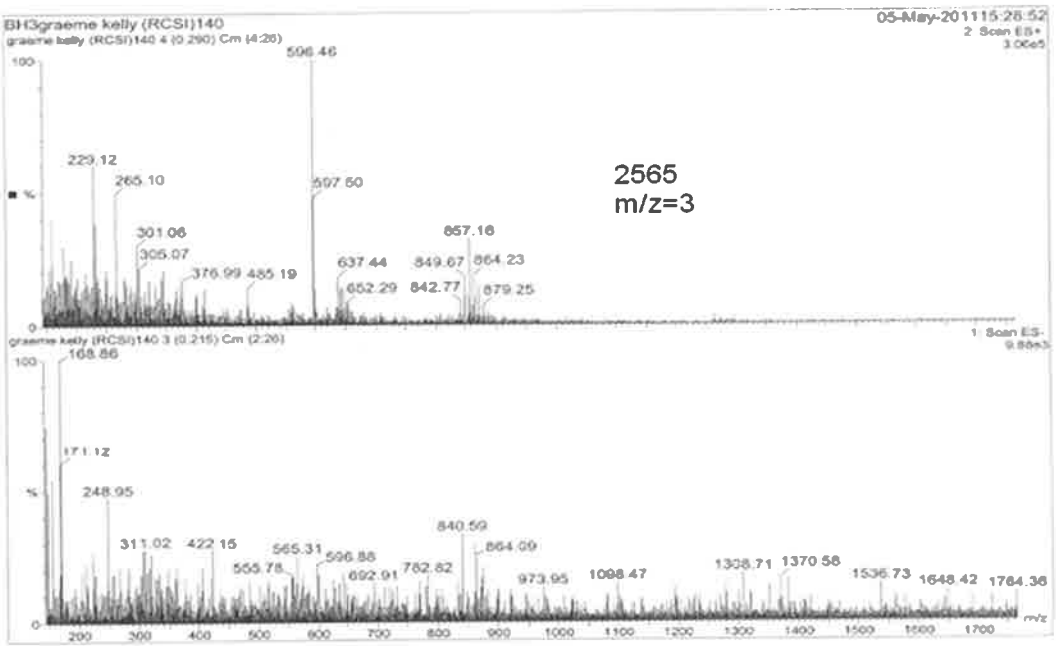
(32) 5.5.1.4 MS (βAla) Ac-BH3-Buforin-NH_2



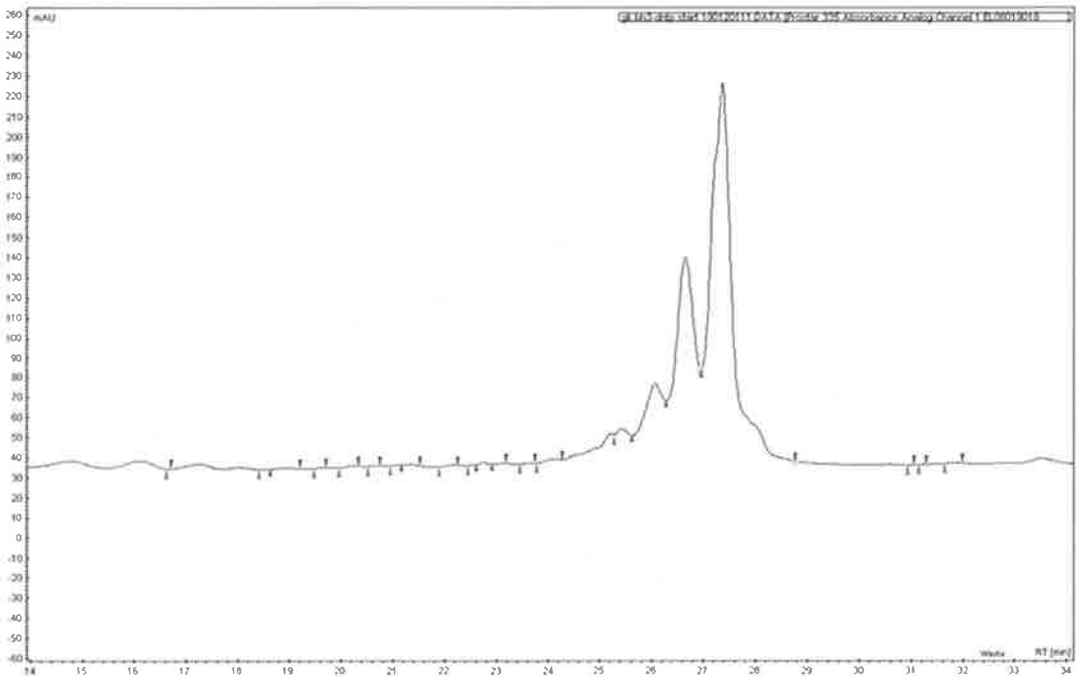
(33) 5.5.1.4 HPLC (βAla) Ac-BH3-Buforin-NH_2



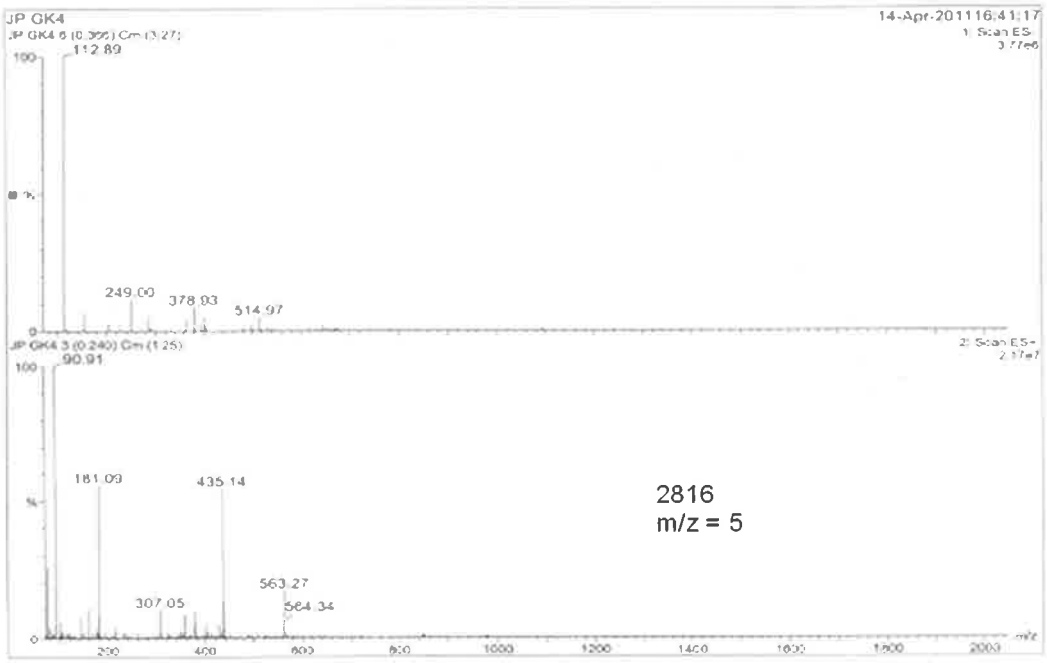
(34) 5.5.2.1 MS Ac-Cys-Ahx-BH3-NH_2



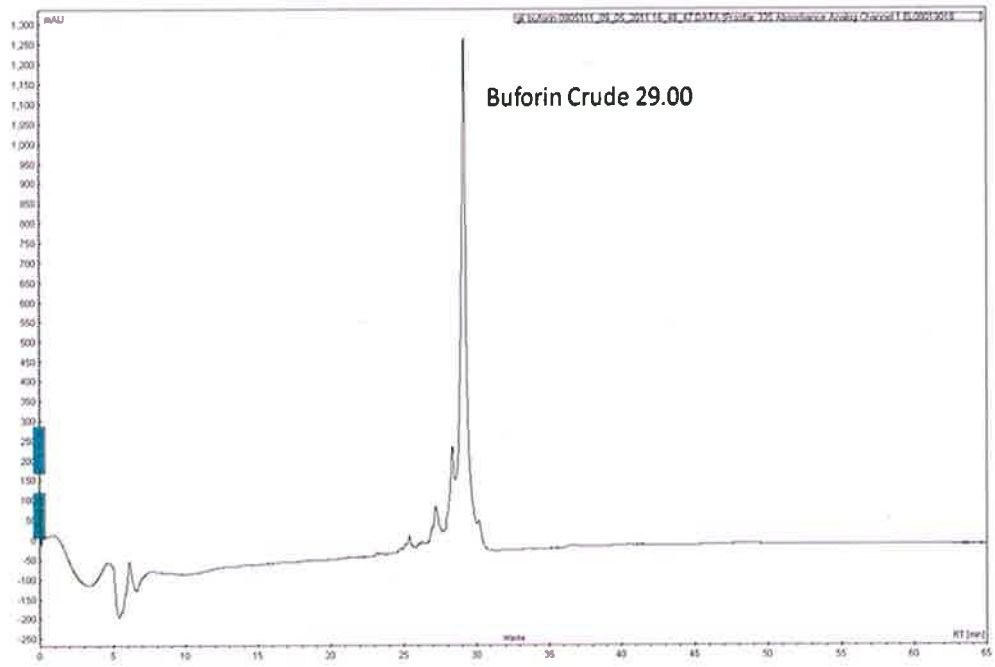
(35) 5.5.2.1 HPLC Ac-Cys-Ahx-BH3-NH_2



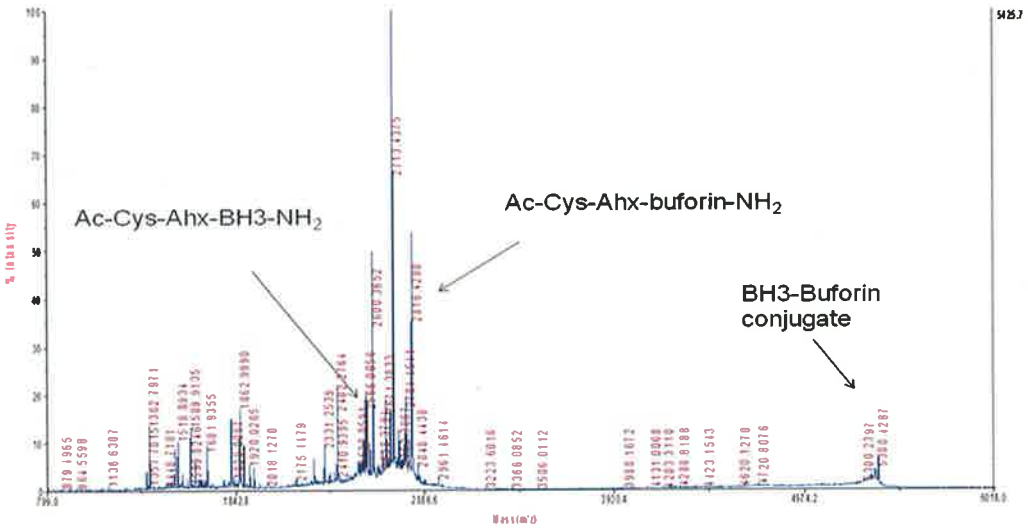
(36) 5.5.2.2 MS Ac-Cys-Ahx-Buforin-NH_2



(37) 5.5.2.2 HPLC Ac-Cys-Ahx-Buforin-NH_2



(38) 5.5.2.4 MS (Ahx) Ac-BH3-Buforin-NH_2



(39) 5.5.2.4 HPLC (Ahx) Ac-BH3-Buforin-NH_2